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Anti-relapse activity of WR 238605 in a shorter 3 dose regimen has been established which would be operationally more acceptable. WR 238605 has also exhibited significant blood schizontocidal, gametocytocidal and causal prophylactic activities, which can be exploited. A new combination regimen comprising WR 238605 plus halofantrine/desbutyl halofantrine has been evaluated. This combination shows additive response and would be useful for management of chloroquine resistant P.vivax infections. Antihistaminic agents have been found to exert causal prophylactic and suppressive blood schizontocidal activities in rodent model. Cyproheptadine also exerts mefloquine resistance reversal action against P.knowlesi and combination may be useful for treatment of mefloquine resistant cases. Azithromycin has also shown causal prophylactic and blood schizontocidal activity in rodent and simian models. In vitro bioassay for evaluation of antimalarials using synzhronized P.knowlesi was developed. Ex vivo bioassay for estimation of drug concentration in serum has been established using P.knowlesi in vitro. Recombinant IL-12 has shown promising prophylactic activity against P.cynomolgi sporozoite challenge.

14. SUBJECT TERMS
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CDRI-WRAIR COLLABORATIVE PROJECT
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SUMMARY OF THE MAJOR ACHIEVEMENTS

1. CYCLIC TRANSMISSION OF THE MALARIA PARASITES

A. *P. cynomolgi* - Rhesus Monkey model

Plasmodium cynomolgi B is being maintained by cyclic transmission through *An. stephensi* mosquitoes and on an average the parasite undergoes a complete monkey-mosquito-monkey cycle in 40-45 days. The parasite has been maintained through 130 cyclic passages through *An. stephensi*.

B. *P. yoelii nigeriensis* (N-67) Swiss Mice model

The cyclic transmission of the rodent malaria parasite *P. yoelii nigeriensis* through *An. stephensi* mosquitoes has also been established. Golden hamster has been found to be a suitable host for infective blood meal and gametocyte production for infection of mosquitoes, and maintenance of cyclic passage of this parasite.

2. REVALIDATION OF CHLOROQUINE AND PRIMAQUINE CURATIVE DOSES AGAINST *P. CYNOMOLGI* B

The curative blood schizontocidal dose of chloroquine (3 mg/kg base x 7 days), and causal prophylactic (1.78 mg/kg x 3 days) and anti-relapse curative doses of primaquine (1.00 mg/kg base x 7 days) have been revalidated and no escalation in curative doses established since 1982 has been observed. The protocols for blood schizontocidal test, causal prophylactic test, anti-relapse test, gametocytocidal/sporontocidal efficacy tests using *P. cynomolgi* B have been maintained operational during the tenure of the project.

3. BLOOD SCHIZONTOCIDAL CURATIVE DOSE OF CHLOROQUINE IN THE SHORTER THREE DOSE REGIMEN

Curative dose of chloroquine has also been determined using shorter three dose treatment schedule and the dose of 10.0 mg base/kg x 3 days by oral route has been found to be curative against *P. cynomolgi* B.

4. BLOOD SCHIZONTOCIDAL ACTIVITY OF ANTIMALARIALS

The curative blood schizontocidal dose of mefloquine, halofantrine and WR 242511 has been established against *P. cynomolgi* B in rhesus monkey model.

A. Mefloquine: The blood schizontocidal activity of mefloquine was evaluated against trophozoite induced *P. cynomolgi* B infection in rhesus monkeys and dose of 10 mg/kg x 7 days, administered orally was curative.

B. Halofantrine: Halofantrine at 10 mg/kg x 7 dose (oral) schedule was curative against blood induced *P. cynomolgi* infection.

C. WR 242511: The blood schizontocidal dose of WR 242511, a 5 methoxy 8-aminoquinoline against *P. cynomolgi* B was determined at 1.00 mg/kgx7 day.

5. ADDITIONAL ANTIMALARIAL DATA WITH COMPOUND WR 238605

Compound WR 238605 identified under CDRI-WRAIR collaborative programme has been selected by Walter Reed Army Institute of Research for Phase II clinical trials. This compound is a potential anti-relapse antimalarial which may eventually replace primquine. In the *P. cynomolgi* rhesus monkey model, this compound has shown 7-10 fold better therapeutic activity compared to primaquine and compound is safe for clinical trials.

A. Blood schizontocidal activity of WR 238605

Additional blood schizontocidal data has been obtained for compound WR 238605 against two simian malaria parasites namely *P. cynomolgi* B and *P. fragile*

and the new compound has shown 10 fold better blood schizontocidal activity than primaquine.

B. Radical curative activity of WR 238605 in the shorter three dose regimen

Compound WR 238605 was evaluated for anti-relapse activity using three dose treatment regimen and a dose of 1.00 mg/kg x 3 days was found to be curative.

C. Gametocytocidal activity of WR 238605

Compound WR 238605 has also shown significant gametocytocidal activity at 2 mg/kg single dose against *P. cynomolgi* B.

6. EVALUATION OF ALTERNATE REGIMENS FOR MANAGEMENT OF CHLOROQUINE RESISTANT *P. VIVAX* CASES

With the establishment of foci of chloroquine resistant *P. vivax* in several geographical regions, the management of this parasite is likely to pose problems in the coming years. Halofantrine and mefloquine are the alternate drugs which can possibly replace chloroquine as the blood schizontocidal agent. Compound WR 238605 is undergoing Phase II clinical trials as a replacement drug for primaquine, as the tissue schizontocidal agent. This new compound has shown improved efficacy and better half-life than primaquine in animal studies carried out earlier at CDRI. The rational for undertaking present study was to evaluate the compatibility of two alternate blood schizontocides, namely halofantrine/mefloquine with WR 238605 for management of chloroquine resistant *P. vivax* cases.

A. Combination studies with halofantrine and WR 238605

(i) Blood schizontocidal activity : Halofantrine shows curative blood schizontocidal activity against blood induced *P. cynomolgi* B infection at 10.0 mg/kg, while compound WR 238605 is also curative at 3.16 mg/kg dose in the standard 7 day blood schizontocidal test. Co-administration of WR 238605 at

0.316 mg/kg in combination with halofantrine at 3.16 mg/kg were found to be curative thereby indicating an additive or possibly synergistic effect of the combination on the blood stages of the parasite. The study shows that in combination the curative dose of halofantrine is reduced by one-third, and that of WR 238605 by one-tenth.

(ii) **Anti-relapse activity:** Combination studies carried out with the above antimalarials for anti-relapse activity against sporozoite induced *P. cynomolgi* B infection also showed that concurrent administration of 0.316 mg/kg WR 238605 (effective anti-relapse dose) along with 3.16 mg/kg halofantrine was curative as evidenced by absence of any relapse in the treated monkeys. The results show that halofantrine does not antagonise the anti-relapse activity of WR 238605 in the simian model and clinical trials with this combination could lead to a drug-regimen for the radical cure in chloroquine resistant *P. vivax* areas.

B. Combination studies with mefloquine and WR 238605

(i) **Blood schizontocidal activity:** Co-administration of 5.62 mg/kg mefloquine and 0.316 mg/kg WR 238605 was curative against blood stages of *P. cynomolgi* indicating additive response of the two components.

(ii) **Anti-relapse activity:** Concurrent administration of 0.316 mg/kg of WR 238605 and 5.62 mg/kg mefloquine also showed radical curative activity against sporozoite induced infections of *P. cynomolgi*, thus indicating the compatibility of the two agents for treatment of relapses. The study clearly establishes that the blood schizontocide chloroquine can be replaced by mefloquine in radical curative test. Clinical trials with mefloquine + WR 238605 combination in chloroquine resistant *P. vivax* areas are warranted.

7. ANTIHISTAMINES AS NEW CLASS OF ANTIMALARIALS:

Cyproheptadine and Ketotifen have shown causal prophylactic activity at 5mg/kg dose x 3 days (-1, 0 +1) against P.yoelii sporozoite challenge. Terfenadin at 50mg/kg dose also protected mice against P.yoelii sporozoite challenge. This is the first report on efficacy of antihistaminic agents (5HT, receptor antagonists) for growth inhibition of pre-erythrocytic stages of any malaria parasite. Cyproheptadine also showed suppressive blood schizontocidal activity at 20mg/kg against blood induced P.yoelii infection.

8. ANTIMALARIAL SPECTRUM OF AZITHROMYCIN:

Azithromycin has shown curative blood schizontocidal activity against P.yoelii at 70mg/kg dose administered from day 0-3 or from day 2-5. The curative response was also obtained with 40mg/kg in extended 7 dose (day 0-6) regimen. In causal prophylactic test 50mg/kg dose (day -1 to +1) protected mice against P.yoelii sporozoite challenge. In comparison, erythromycin did not show either of the above activities upto 405 mg/kg dose in identical regimens. Against the simian parasite, 25mg/kg x 7 days cured trophozoite induced P.cynomolgi infection. In the prophylactic test, prepatent period of azithromycin (25 mg/kg x 9 doses) treated monkeys was significantly extended after challenge with P.cynomolgi sporozoites. Azithromycin did not exhibit any hypnozoitocidal effect with 25 mg/kg x 7 day regimen.

9. DRUG RESISTANT STRAINS FOR RESISTANCE REVERSAL STUDIES

(a) **Simian malaria**

The following sub-lines of *P. knowlesi* W_1 have been initiated with a view to establish stable drug resistance.

(1) Chloroquine resistant strain: Efforts are continuing to establish chloroquine resistant strain of *P. knowlesi*, but so far resistance to chloroquine has not been established though the parasite was exposed to sub-curative doses of the drug *in vivo* for over **a one year period.**

(2) Mefloquine resistant strain has been developed and it can tolerate mefloquine up to 80 mg/kg x 3 doses. This strain will be useful for pre-clinical evaluation of mefloquine resistance reversal agents such as penfluoridol and other potential reversal agents. The mefloquine resistant *P. knowlesi* has been cryopreserved.

b) Rodent malaria

(i) The following drug resistant lines of rodent malaria parasite *P. berghei* have been cryopreserved.

1. Chloroquine resistant strain (resistant up to 128 mg/kgx4)
2. Mefloquine resistant strain (resistant up to 128 mg/kgx4)
3. Quinine resistant strain (resistant up to 400 mg/kgx4)

(ii) A multiple resistant strain of *P. yoelii nigeriensis* resistant to chloroquine (128 mg/kgx4), mefloquine (128 mg/kg x 4) and quinine (400 mg/kgx 4) has been cryopreserved. This strain has been used for resistance reversal studies as it produces 100% lethal infection.

(iii) Additional drug resistant strains of mosquito transmissible *P. yoelii nigeriensis* (N-67) have been selected in the Swiss mice model.

(a) Chloroquine resistant strain 128 mg/kg

(b) Mefloquine resistant strain 128 mg/kg

(c) Halofantrine resistant strain 128 mg/kg

(d) Pyrimethamine resistant strain 48 mg/kg

The stability of the above resistant strains after transmission through the vector (*A. stephensi*) has been established. The strains would be useful for resistance reversal studies, and would serve as primary *in vivo* screens for resistance reversal activity.

10. STUDIES ON REVERSAL OF DRUG RESISTANCE

Several resistance reversal agents have been published in literature but in most of the studies the reversal effect was observed against *in vitro* cultures of chloroquine resistant *P. falciparum*. Studies have been carried out to validate the resistance reversal effect in drug-resistant rodent malaria model (*P. yoelii nigeriensis*).

A. Resistance reversal studies with multi-drug resistant *P. yoelii nigeriensis*

(i) WR 238605 + chloroquine combination

The marginal extension of MST by 2 days was observed when WR 238605 (0.5 mg/kg) was given together with chloroquine (4.0 or 8.0 mg/kg), as compared to MST of control/chloroquine group suggesting some additive effect of WR 238605 when combined with chloroquine.

(ii) WR 238605 + Mefloquine combination

WR 238605 (at 0.5 mg/kg) did not potentiate the effect of mefloquine against mefloquine resistant strain. However, the combination exerts additive antimalarial effect as shown in the therapeutic (post-treatment) regimen.

(iii) **Verapamil**

Verapamil at higher doses provided a definitive extension of MST when the drug was given together with chloroquine. Studies show a limited chloroquine resistance reversal effect of verapamil in day 2-6 treatment schedule.

iv) **Nifedipine**

Nifedipine at 10-15 mg/kg given with chloroquine 8 mg/kg resulted in extension of mean survival time to 24.7-24.8 days compared to 21.14 days of chloroquine control group, indicating some chloroquine resistance reversal effect of Nifedipine against multiple resistant rodent model used in this study.

v) **Quinidine**

Quinidine in combination with chloroquine exerts a possible additive action and no resistance reversal action was recorded.

B. Resistance reversal studies with mosquito transmissible *P. yoelii nigeriensis* (N-67) Swiss mice

P. yoelii nigeriensis (N-67 strains) resistant to chloroquine and mefloquine were selected after interrupted sub-curative therapy and resistance was found to be stable after transmission through vector.

i) **Verapamil**

Verapamil in combination with chloroquine, mefloquine or halofantrine shows low level of resistance reversal activity as shown by suppression of parasitaemia on day 4.

ii) **Amitryptiline**

Combination of amitryptiline with chloroquine, mefloquine or halofantrine showed only transient suppression of parasitaemia on day 4 and 7 in the combination treated groups.

iii) **Cyproheptadine**

Cyproheptadine has shown promising resistance reversal action against

chloroquine and halofantrine resistant strains as the combination treated animals were completely protected. Cyproheptadine has also significant activity in combination with mefloquine against mefloquine resistant strain.

C. Resistance reversal studies in simian malarial model (*P. knowlesi* rhesus monkey)

Cyproheptadine has been found to show resistance reversal activity against mefloquine resistant *P. knowlesi* in rhesus monkeys. Combination of 20 mg/.kg mefloquine x 3 days plus 10 mg/kg cyproheptadine x 5 days protected the treated monkeys while the two components individually are not curative. This is a promising lead where cyproheptadine has shown resistance reversal action against mefloquine resistant parasite. Mefloquine alone upto 80 mg/kg x 3 days does not cure *P. knowlesi*.

11. *IN VITRO* CULTIVATION AND BIOASSAY FOR ANTIMALARIALS

A. *In vitro* cultivation of *P. falciparum*

In vitro anti-malarial screening protocol against *P. falciparum* has been standardized using Giemsa staining of culture smears to monitor the parasiticidal dose end-point.

B. *In vitro* cultivation of simian parasite *P. knowlesi*

Over the years culture adapted parasites have found major application in evaluation of novel chemotherapeutic agents and drug combinations. One of the major limitations in wider use of *P. falciparum* cultures in the developing countries has been the poor availability of quality human serum which is indispensable for maintaining the continuous cultures. Hence studies were undertaken to standardize *in vitro* model using *P. knowlesi* parasites for evaluation of potential chemotherapeutic agents. The various factors which influence the parasite maturation have been optimized and base line data with reference anti-malarials has been obtained.

C. *In vitro* bioassay for anti-malarials using simian parasite *P. knowlesi*

Short term *in vitro* culture of *P. knowlesi* has been standardized using the candle jar technique and base-line data on ^{3}H-hypoxanthine incorporation at varying concentration of parasitaemia and haematocrit has been obtained using 24 hour incubation period. The application of this model for *in vitro* assay of potential anti-malarials using Giemsa stained blood smears has also been standardised for comparison.

D. Development of *in vitro* anti-malarial assay system using parasite LDH

An *in vitro* system for anti-malarial assay based on possible inhibition of the parasite LDH activity is being standardized using NAD and APAD as cofactors for the LDH biochemical assay. The LDH assay with APAD as substrate has been found to be very sensitive and this could be exploited as an *in vitro* screen for potential blood schizontocides.

E. *In vitro* tissue schizontocidal screening model

For *in vitro* screening of prospective tissue schizontocides, technology to obtain primary monkey hepatocyte cultures and development of exo-erythrocytic stages following inoculation of *P. cynomolgi* B sporozoites has been established. Primaquine at 0.1 μg/ml has been found to inhibit development of primary e-e schizonts.

11. P. FALCIPARUM PARASITE VIABILITY VERSUS DURATION OF DRUG EXPOSURE IN VITRO:

The viability of P.falciparum parasites after exposure to various concentrations of chloroquine and mefloquine for 3-72 hours was monitored. The parasites were washed free of drug after various time intervals and reincubated in fresh medium to determine the viability during subsequent 144 hours. Parasites exposed to chloroquine at 1000ng/ml and 5000ng/ml remained viable upto a maximum exposure time of 36 hours, while 200ng/ml concentration produced total parasiticidal effect after 48 hours. Likewise with mefloquine complete inhibitory effect was observed after exposure to 200ng/ml for 24 hours, while higher concentrations of 1000ng/ml and 5000ng/ml resulted in complete loss of viability even after 3 hours.

12. EX VIVO BIOASSAY OF ANTIMALARIALS:

The in vitro microassay technique has been applied to develop a relatively simple model to bioassay serum drug concentrations of antimalarials in serum samples. The drug equivalent concentrations were determined by multiplying the maximum inhibitory dilution (MID) of test serum and the MIC of drug in vitro against same parasite. The initial studies have been carried out with serum samples collected 1-144 hours after administration of chloroquine (10mg/kg, 20mg/kg, 30mg/kg) or halofantrine (10mg/kg, 20mg/kg, 30mg/kg to naive rhesus monkeys. Calculated chloroquine equivalent values showed a peak of 626ng/ml at 6hr after 10mg/kg dose and peak level of 1252ng/ml at 2 hr after 20 and 30mg/kg dose. Derived halofantrine equivalents showed concentration level of 500ng/ml between 2-6 hours after 20mg/kg dose and 1000ng/ml between 4-12 hours after 30mg/kg dose.

14. *IN VITRO* METHEMOGLOBINS TOXICITY ASSAY

A simple and rapid *in vitro* assay using mastomys erythrocytes has been established to compare the relative toxicity of 8-aminoquinoline antimalarials.

15. MALARIA PROPHYLAXIS WITH RECOMBINANT IL-12 AGAINST *P. CYNOMOLGI* B SPOROZOITE CHALLENGE (COLLABORATION WITH NAVAL MEDICAL RESEARCH INSTITUTE, BETHESDA, DEPARTMENT OF U.S. NAVY)

A single dose of 10 μg/kg of recombinant human IL-12 (rHuIL-12) administered 2 days before challenge with *Plasmodium cynomolgi* sporozoites protected 7 out of 7 rhesus monkeys against malaria. Protection was associated with increase in circulating IFN-r and IFN-r, IL-6, IL-10, IL-12, IL-15 and TNF-α mRNA. It is believed that IL-12 protects monkeys through IFN and nitric oxide dependent elimination of infected hepatocytes. This first report of IL-12 induced protection of primates against an infectious agent supports assessment of rHuIL-12 for immunoprophylaxis against human malaria.

PROGRESS OF WORK (FEBRUARY 1993-FEBRUARY, 1998)

1. CYCLIC TRANSMISSION OF MALARIA PARASITES

A. Cyclic passage of *P. cynomolgi* B

The transmission of simian parasite *P. cynomolgi* through the vector has been maintained and the parasite has undergone 130 sequential passage since the initiation of the WRAIR-CDRI collaborative project in 1982. The details of serial passages (87-130) maintained during the period of report are summarized in Table 1.

The parasite has given high infectivity in *Anopheles stephensi* (colony bred). The insectary is maintaining 2000-3000 pupae/day under standard insectary conditions. Adequate numbers of sporozoites can be produced for accomplishing the tasks involved in prophylactic and radical curative tests. Prepatent period in rhesus monkeys after sporozoite inoculation ($0.26x10^6$ to $1.54x10^6$) has been recorded to range between 7-9 days.

B. Cyclic passage of *P. yoelii nigeriensis* (N-67)

A gametocyte producing strain of *P. yoelii nigeriensis* was obtained from Malaria Research Centre, Delhi and the optimum conditions for the transmission of this parasite through *A. stephensi* mosquitoes have been established. Hamster has been found to be a suitable host for obtaining gametocytes for infectivity studies. This model will be useful for prophylactic studies involving drug resistant parasites. (Fig. 1A, 1B).

2. REVALIDATION OF CHLOROQUINE AND PRIMAQUINE CURATIVE DOSES

A. Chloroquine blood schizontocidal dose

The curative dose of chloroquine against blood induced *P. cynomolgi* B infection was established as 5 mg base/kg x 7 days (oral). The treated monkeys

are observed for 60 days after the end of treatment and absence of any recrudescence during this period indicates curative activity. The dose of chloroquine was revalidated several times during the last 4 years and no escalation in curative dose has been recorded.

Three day regimen: Three day regimen of chloroquine was evaluated against blood induced infection of *P. cynomolgi* B using 5.0, 7.5 and 10.0 mg/kg doses of chloroquine (base) administered orally for three consecutive days. Results in Table 2 show that 10.0 mg/kg was the curative dose, 7.5 mg/kg was curative in one out of two monkeys and 5.0 mg/kg failed in both the monkeys. The monkeys which showed recrudescence in the above study were again treated at 7.5 mg/kg and 10.0 mg/kg and both these doses were curative.

B. Primaquine prophylactic and radical curative dose

The causal prophylactic dose of primaquine (1.78 mg base/kgx3 days) was revalidated against sporozoite induced *P. cynomolgi* B infection in 2 monkeys and both the monkeys were cured (Table 3).

Radical curative dose of primaquine (1 mg/kg base x 7 days) was revalidated in 2 monkeys each during 86th and 90th serial passages and dose was found to be curative. The lower dose 0.316 mg/kgx7 days used during 86th serial passage was not curative as expected and monkeys relapsed on day 29 and 37 (Table 4 and 5).

The curative blood schizontocidal dose of chloroquine and causal prophylactic and radical curative doses of primaquine, have shown no escalation during the last 15 years.

3. BLOOD SCHIZONTOCIDAL ACTIVITY OF MEFLOQUINE, HALOFANTRINE AND WR 242511

A. Blood schizontocidal activity of Mefloquine

The blood schizontocidal activity of mefloquine was evaluated in 2 monkeys each at 3.16 mg/kg, 10 mg/kg and 31.6 mg/kgx7 days. The lower dose of 3.16

mg/kg failed to clear the parasitaemia in both the monkeys while parasite clearance was recorded in 72-96 hours in monkeys treated at higher doses. There was no recrudescence in any of the monkeys treated at 10.0 and 31.6 mg/kg till 60 days (Table 6). The dose of 10 mg/kg was revalidated in 2 naive monkeys and both were protected (Table 7).

B. Blood Schizontocidal activity of Halofantrine

The blood schizontocidal activity of Halofantrine was evaluated in 2 monkeys each at 3.16 mg/kg, 10.00 and 31.6 mg/kgx7 days. The parasite clearance in all the monkeys was observed between 48-72 hours. The lowest dose of 3.16 mg/kg was not curative as indicated in Table 8. Monkeys at the higher dose i.e. 10.00 and 31.6 mg/kgx7 days were cured and did not show any recrudescence. Activity at 10 mg/kgx7 days was revalidated in 2 monkeys (Table 9), and it was found to be curative. Further tests were carried out at 5.6 mg/kgx7 days dose schedule in four monkeys, and the compound was curative at this dose in three out of four monkeys, while the fourth showed recrudescence. Test carried out at 10 mg/kgx7 days, was curative in both the monkeys (Table 10).

C. Blood schizontocidal activity of WR 242511

The blood schizontocidal activity of WR 242511 was evaluated in 2 monkeys each at 0.316 mg/kg, 1.00 mg/kg and 3.16 mg/kgx7 days. The lowest dose of 0.316 mg/kg was not curative as indicated in Table 11. Monkeys at the doses of 1.00 and 3.16 mg/kgx7 days were cured and have not shown recrudescence during 60 days observation period. Revalidation of 1 mg/kgx7 days dose showed that the dose was curative in two monkeys (Table 12).

In view of the sporadic emergence of chloroquine resistant *P. vivax* parasites, the treatment of resistant cases would need the shifting of chloroquine therapy to an alternate blood schizontocide for use as companion drug with the radical curative agent like primaquine or the new compound WR 238605 which is under clinical phase II trials at Walter Reed. Amongst the alternate blood

schizontocides which can replace chloroquine include mefloquine and halofantrine. With a view to establish their efficacy, the data generated with these compounds clearly show that mefloquine and halofantrine are curative as blood schizontocides at 10 mg/kgx7 days.

4. ADDITIONAL ANTIMALARIAL DATA WITH COMPOUND WR 238605

Preclinical evaluations carried out earlier at CDRI with compound WR 238605 had demonstrated this new compound to be 7-10 fold more active as causal prophylactic or radical curative drug. The radical curative dose was established at 0.316 mg/kgx7 days in the *P. cynomolgi* rhesus monkey model and the data was pivotal to the design of Phase I and Phase II clinical investigations now being carried out in Thailand by WRAIR. Additional studies have been carried out with this compound to establish its additional spectrum of activity as blood schizontocidal agent and gametocytocidal agent. Besides, the radical curative dose of this compound using shorter 3-dose regimen has also been established.

A. Blood schizontocidal activity against *P. cynomolgi* B and *P. fragile*

The blood schizontocidal activity of compound WR 238605 has been evaluated against two simian parasites *P. cynomolgi* B and *P. fragile*. Results in Table 13 show that against *P. cynomolgi* B infection, 10 out of 12 monkeys were protected at 1 mg/kg dosex7 days. All the 6 monkeys treated at 3.16 mg/kgx7 were also cured. In comparison primaquine was not curative in any of the 4 monkeys at 3.16 mg/kgx7 and in 3 out of 4 monkeys at 10 mg/kgx7 days. Likewise, against *P. fragile* infection, compound WR 238605 cured 10 out of 11 monkeys at 1 mg/kg and all the 4 monkeys at 3.16 mg/kgx7 days. Primaquine protected 1 out of 3 monkeys at 3.16 mg/kgx7 and 2 out of 3 monkeys at 10 mg/kg dose x 7 days. The study concludes that compared to the primaquine, compound WR 238605 has shown 10 fold higher blood schizontocidal activity against *P. cynomolgi* B and *P. fragile* infections in rhesus monkey models.

B. Gametocytocidal activity of WR 238605 against *P. cynomolgi* B

For the gametocytocidal test, batches of 3-4 day old *An. stephensi* were allowed to feed on *P. cynomolgi* infected rhesus monkeys at appropriate gametocytaemia level. Our earlier studies have shown that the sequential feeding of healthy mosquitoes on 3-4 consecutive days during the declining phase of the secondary asexual peak parasitaemia gave consistently good infectivity. One hr after the control (pre-treatment) feeding, compound WR 238605 was administered to the monkeys at 1.0, 2.0 and 4.0 mg(base)/kg in a single dose by oral route. Post-treatment feeding of batches of healthy mosquitoes was done at different times (6-8 hr). Mosquitoes were maintained at 26±1°C under optimal insectary conditions. The infectivity rate and the oocyst counts were recorded on day 8. Mosquitoes were further maintained in the insectary upto day 15 to determine the formation of sporozoites in the experimental batches.

RESULTS

The gametocytocidal activity of WR 238605 was evaluated in 7 rhesus monkeys and the pre-treatment mosquito infectivity results for these monkeys show that the oocyst number of different batches ranged from 17.13±10.01 to 35.32±13.34 and the percent infectivity varied between 64.10 to 86.49% (Table 14). Sequential mosquito feedings on three monkeys treated at 1.00 mg/kg dose showed that there was no significant reduction in oocyst number and the percent infectivity in +6 hr mosquito batches for all the three monkeys and in +24 hr post-treatment batches for two out of three monkeys when compared to the corresponding control feeding at -1 hr. Salivary gland dissections of the mosquitoes from these batches on day 15 showed the presence of sporozoites, thus indicating that oocysts completed normal sporogonic development. No oocysts were observed over the midguts from mosquitoes fed at +48 hr after drug administration nor were any sporozoites seen in their salivary glands.

Identical results were obtained in the efficacy tests at 2.0 mg/kg in 3/3 monkeys and at 4.0 mg/kg in one monkey. The mosquito batches fed at +6 hr post-treatment showed no significant alteration in the oocyst numbers, and these oocysts were able to complete the sporogonic cycle as indicated by the presence of sporozoites in salivary glands on day 15-16. The mosquito batches fed on these monkeys at +24 hr did not develop any oocysts nor were any sporozoites demonstable in their salivary glands.

C. **Shorter three dose regimen for radical curative activity**

Compound WR 238605 had been earlier found to show anti-relapse activity at 0.316 mg/kg dose in the seven day regimen. Studies were carried out to determine the curative dose of the compound in "Three dose Regimen". Two monkeys each were treated at 0.50 mg/kg, 1.0 mg/kg and 2.00 mg/kg x 3 days. Monkeys treated at 0.50 mg/kg relapsed on days 25 and 43 while monkeys treated at higher doses were protected. In the second experiment, 3 monkeys treated with 0.75 mg/kg x 3 days were also protected (Table 15).

D. **Radical curative efficacy without companion blood schizontocidal agent:**

Since compound WR 238605 apart from being a potential anti-hypnozoitocidal agent has also shown promising blood schizontocidal activity, tests were carried out to determine response of this compound against established sporozoite induced infections with P.cynomologi B. In the first experiment, three monkeys each were treated with 1.00 mg/kg and 3.16 mg/kg dose x 7 days without companion blood schizontocidal agent (Table 16). None of the six treated monkeys showed any relapse. In the 2nd experiment, dose of 1.00 mg/kg was revalidated in another three monkeys, thus confirming the curative activity (Table 17).

5. COMBINATION STUDIES WITH COMPOUND WR 238605 AND HALOFANTRINE

In view of the sporadic emergence of chloroquine resistant *P. vivax*, the treatment of resistant cases would need the shifting of chloroquine therapy to an alternate blood schizontocide for use as companion drug with the radical curative agent like primaquine or the new compound WR 238605 which is under clinical phase I trials at Walter Reed. Amongst the alternate blood schizontocides which can replace chloroquine include mefloquine and halofantrine. With a view to establish their efficacy, the data generated with these compounds clearly showed that mefloquine and halofantrine are individually curative as blood schizontocides at 10 mg/kgx7 schedule against blood induced *P. cynomolgi* infection in rhesus monkeys. The reference blood schizontocidal drug chloroquine is curative in this model at 3.00 mg/kg x 7 day.

Further studies have been carried out using halofantrine in combination with the anti-relapse antimalarial WR 238605 in both the blood schizontocidal test and the radical curative test with a view to see whether the combination has additive/antagonistic effect.

A. Blood schizontocidal activity of WR 238605 and halofantrine combination

Studies with these compounds when used individually had shown that compound WR 238605 is curative at 3.16 mg/kgx7 days and halofantrine is curative at 10.0 mg/kgx7 days. Concurrent administration of WR 238605 at 0.316 mg/kg and halofantrine at 3.16 mg/kgx7 days protected two out of two monkeys, while WR 238605 at 0.316 mg/kg in combination with halofantrine at 1.00 mg/kg was not curative in any of the two monkeys (Table 18). Results indicate that the combination shows additive/synergistic effect as the curative doses of the components in the combination have been lowered by 10 and 3 fold respectively (Table 19).

B. Radical curative activity of WR 238605 and halofantrine combination

To evaluate the anti-relapse efficacy of WR 238605 in combination with halofantrine as the companion blood schizontocide, two monkeys each were treated with a combination of WR 238605 and halofantrine at 0.316 mg/kg + 3.16 mg/kg, 0.316 mg/kg+5.62 mg/kg and 0.316 mg/kg+10.0 mg/kgx7 days respectively. Follow up of these monkeys till 100 days showed that none of the monkeys relapsed thus indicating the curative efficacy of the doses (Table 20). In the second experiment, compound WR 238605 at 0.316 mg/kg dose was evaluated in combination with halofantrine at 1.78 mg/kg, 3.16 mg/kg and 5.62 mg/kg doses in two monkeys each. While one monkey at 0.316 mg WR 238605 + 1.78 mg/kg halofantrine relapsed on day 26, the other five monkeys were cured (Table 21). One monkey treated with compound WR 238605 alone at 0.316 mg/kg relapsed on day 26. Additional two monkeys treated with Wr 238605 at 0.1 mg/kg and halofantrine at 10 mg/kg also relapsed on days 13 and 15. The efficacy of combination of 0.316 mg/kg 238605 + 3.16 mg/kg halofantrine was revalidated in 2 monkeys in the third experiment (Table 22) and the dose was again found to be curative. The summarized data of combination studies is presented in Table 23 and results show that halofantrine does not antagonize with the anti-relapse activity of compound WR 238605.

C. Radical curative activity of WR 238605 and Desbutyl halofantrine combination:

The curative dose established using WR238605 and halofantrine combination (0.316 mg/kg + 3.16 mg/kg respectively) was again found curative when Desbutyl halofantrine was used instead of halofantrine (Table 24). The results show that Desbutyl halofantrine also shows additive response when used concurrently with WR 238605.

6. COMBINATION STUDIES WITH COMPOUND WR 238605 AND MEFLOQUINE

A. Blood schizontocidal activity of mefloquine and WR 238605 combination

The blood schizontocidal efficacy of mefloquine with a combination of WR 238605 was calculated at two dose levels. Two monkeys treated with 3.16 mg/kg mefloquine plus 0.316 mg/kg WR 238605 recrudesced on day 25 and 26, while two monkeys treated with combination of 5.62 mg/kg mefloquine and 0.316 mg/kg 238605 were protected. Another two monkeys treated with mefloquine alone at 5.62 mg/kg dose also recrudesced on days 12 and 17. The results suggest additive response of the two antimalarials (Table 25).

B. Radical curative activity of WR 238605 using mefloquine as the companion blood schizontocide

For the radical curative test four monkeys were administered 0.316 mg/kg 238605. Mefloquine at two dose levels (viz. 5.62 mg/kg and 10.0 mg/kg) was administered as the companion blood schizontocide using two monkeys for each dose. The results showed that WR 238605 (0.316 mg/kg) plus 10 mg/kg mefloquine, as well as WR 238605 (0.316 mg/kg) plus 5.62 mg/kg mefloquine, were curative as antirelapse regimen (Table 26). Mefloquine alone at 10 mg/kg dose showed relapse on days 11 and 12, as expected. The results show that mefloquine does not antagonise the antirelapse efficacy of compound WR 238605.

7. ADDITIONAL DATA WITH COMPOUND WR 242511

Radical curative efficacy without companion blood schizontocidal drug:

In our previous studies compound WR 242511 was demonstrated to possess blood schizontocidal efficacy against trophozoite induced infections with P.cynomolgi B and dose of 1.00 mg/kg x 7 days was established as the curative dose. In continuation to those observations, the radical curative activity of WR 242511 without companion blood schizontocidal agent was determined in three monkeys and dose of 1.00 mg/kg (x 7 days) was again found to show the curative response (Table 27).

8. ANTIHISTAMINICS AS A NEW CLASS OF ANTIMALARIALS

a. Blood Schizontocidal Activity:

Four anti-histaminic drugs namely Terfenadine, Mebhydrolin, CDRI 73/602 (anti-histaminic compound under phase II clinical trials), and cyproheptadine, were evaluated for their antimalarial potential against *P. yoelii nigeriensis* resistant to chloroquine, mefloquine and quinine. The results presented in Table 28 show that the cyproheptadine possesses exceptionally high anti-malarial activity at 20 mg/kg dose, giving 50% survival of the mice beyond 21 days. The mean survival time of the control group was 7.25 days, while the 20 mg/kg cyproheptadine extended the MST to more than 16 days. This is a new lead and it is proposed to get some new analogues of cyproheptadine as well as other antihistaminics and 5-HT antagonists tested for their antimalarial activity. The other three antihistaminics tested did not show any antimalarial action (Table **28**).

b. Casual Prophylactic Activity:

The causal prophylactic activity of five tricyclic antihistaminic agents (histamine H_1-receptor)antagonists) namely cyproheptadine, ketotifen, loratadine, azatadine and terfenadin was evaluated in experimental murine malarial model using sporozoite induced infections with P.yoelii nigeriensis (N-67). The results showed that cyproheptadine 5 mg/kg , ketotifen 5 mg/kg and terfenadine 50mg/kg showed 100% prophylactic activity as none of the mice under these regimens developed patent infection. (Table 29) Efficacy tests with lower doses showed that 2 out of 10 mice treated with cyproheptadine at 2.5 mg/kg were cured and the remaining mice became patent between day 5-7. Ketotifen exhibited 50% protection at 2.5 mg/kg and patency in other 5 mice was delayed to between 7-10 days. The prepatent period was also extended in mice treated at 1.25 mg/kg dose with the above two agents as also in groups

treated with terfenadine at 25 and 12.5 mg/kg dose levels. Azatadine and loratadine were not curative up to 50 mg/kg dose tested, however, the prepatent period was extended over control values. Mice treated with Primaquine at 32 mg/kg and pyrimethamine at 0.1 mg/kg were cured while all the control mice became patent between day 3-5.

In order to rule out the residual effect of these drugs against emerging exo-erythrocytic merozoites and intraerythrocytic stages, groups of mice were adminstered identical regimens of cyproheptadine (5mg/kg), ketotifen (5mg/kg) and terfenadine (50 mg/kg) for three consecutive days and animals were challenged with 1×10^5 blood stage parasites, 24 hours after the last dose to coincide with the time of maturation and rupture of intrahepatic schizonts. No significant difference in the course of parasitaemia in drug treated and control animals showed that antihistaminic agents did not exert residual effect.

c. **Radical curative test with cyproheptadine:**

In view of the novel causal prophylactic activity of cyproheptadine identified in the murine model, this agent was also evaluated for anti-relapse activity against P.cynomolgi -monkey model. Two monkeys carrying established sporozoite induced infection were administered cyproheptadine at 5 mg/kg dose (x 7 days) along with chloroquine (5 mg/kg). Although the relapse occurred in both the monkeys there was significant delay in the relapse interval indicating partial response of the test agent (Table 30).

Blood schizontocidal activity against P. yoelii nigeriensis (N-67)

The response of infection after treatment with azithromycin at 15, 45, 70, and 135 mg/kg as well as erythromycin at 45, 135 and 405 mg/kg is presented in Table-1 . Azithromycin showed curative blood schizontocidal activity at 70 mg/kg dose and lower doses produced well marked dose response. None of the mice treated with 70 and 135 mg/kg developed patent infection. Treatment with 45 mg/kg dose delayed the patency to beyond day 7 and only low level of infection appeared between day 10 and 19 Three out of 12 mice under this regimen did not develop patent infection and all the 12 mice survived. The day 4 parasitaemia was significantly suppressed after treatment with 15 mg/kg dose as compared to the level in control group. Five out of 12 mice survived the infection while mean survival time of remaining seven mice was significantly extended. Untreated control mice developed rapid increase in infection and all the mice died with mean survival time of 10.00±0 57 days. In comparison none of the erythromycin treated mice was cured upto 405 mg/kg dose, though day 4 parasitaemia was suppressed. Two, four and six mice survived respectively after treatment at 45, 135, and 405 mg/kg(Table- 31).

Further studies to determine therapeutic efficacy of azithromycin against established infection revealed that 70 mg/kg dose was curative even if treatment was initiated two days post inoculation when initial parasitaemia ranged between 2-4%. The treatment with lower dose of 45 mg/kg initially cleared the parasitaemia in all the mice though low level of infection appeared in 7 out of 12 mice between days 16 and 19(Table-. 32).

In subsequent studies to determine whether curative response with azithromycin could be obtained at lower doses by extending the duration of treatment, the response of 4 day (0-3) and 7 day

(0-6) treatment regimens was compared at 10, 20 and 40 mg/kg dose levels. Results in Table-33 show that the parasite level was lower after 10 mg/kg x7 day schedule compared to corresponding 4 day treated group and the difference between the two regimens was well marked at 20 mg/kg dose level. Treatment at 40 mg/kg dose resulted in clearance of parasitaemia after 4 dose regimen, though recrudescence was recorded after day 13. On the other hand 40 mg/kg x7 dose schedule was curative as none of the mice developed patent infection.

Comparative ED_{50}/ ED_{90} values of azithromycin and erythromycin are given in Table-4. In addition the results have been compared with another antibiotic doxycycline. Results show that on ED_{90} basis azithromycin was 30.66 fold more active than erythromycin and the values were comparable to that of doxycycline (Table-34).

Causal prophylactic activity against P. yoelii nigeriensis (N-67)

The results of causal prophylactic activity of azithromycin and erythromycin against challenge with P. yoelii nigeriensis sporozoits are presented in Table-35. Mice treated with azithromycin at 50 mg/kg and 100 mg/kg did not develop patent infection as parasites were not detected in blood smears upto day 28. All the mice treated with erythromycin at 135 and 405 mg/kg as well as vehicle control animals developed patent infection between day 4-7. Primaquine and pyrimethamine used as reference drugs elicited causal prophylactic activity at 32 and 0.1 mg/kg respectively.

In order to rule out the residual effect of azithromycin on erythrocytic stage parasites, a group of 6 mice was administered 50 mg/kg azithromycin in identical four dose regimen and challenged with $1x10^5$ blood stage parasites, four hours after the last dose to coincide with the time of maturation and rupture of exo-erythrocytic schizonts. There was no significant difference in the course of parasitaemia

in treated and control groups and all the mice died with high parasitaemia by day 10 thus indicating that azithromycin did not exert any residual effect.

Blood schizontocidal activity against P. cynomolgi B in rhesus monkeys.

Blood schizontocidal activity of azithromycin was evaluated at 25 mg/kg dose in 4 monkeys inoculated with blood stage parasites of P. cynomolgi. The initial parasitaemia at the start of treatment ranged between 4900 to 7635/mm^3. The parasitaemia level increased initially for 72-96 hrs. in spite of treatment with azithromycin, however, parasites were cleared from circulation by day 7 and recrudescence was not recorded in any of the 4 monkeys during the subsequent follow up for 60 days (Fig 2).

Causal prophylatic activity against P. cynomolgi B in rhesus monkeys.

Rhesus monkeys inoculated with sporozoites of P. cynomolgi were treated at various dose levels ranging between 6.25–25.0 mg/kg for 9 days (Table36).The placebo treated monkeys became patent on day 10 and 11 while patency in one monkey treated with 6.25 mg/kg azithromycin was delayed to day 21. Two monkeys treated at 12.5 mg/kg dose became patent on day 23 and 29 and in another three monkeys treated at 25 mg/kg dose, patency was further delayed to day 33, 39 and 47. Primaquine at 1 mg/kg dose was curative, while two monkeys treated with pyrimethamine at 10 mg/kg dose became patent on days 33 and 39

Antirelapse activity against P. cynomolgi B in rhesus monkeys.

The antirelapse efficacy of azithromycin was evaluated at 25 mg/kg dose schedule in two monkeys .Both the monkeys relapsed on day 19 and 20 post treatment. The chloroquine control monkey relapsed on day 17 (Table- 37).

10. DRUG RESISTANT SIMIAN MALARIA STRAINS

A. Selection of chloroquine resistant strain of *P. knowlesi*

i) Selection by relapse technique

Attempts were made to select a chloroquine resistant strain of *P. knowlesi* W_1 by sequential treating the infected monkeys at high parasitaemia level and the surviving parasites were inoculated into naive monkeys 24-48 hr after drug exposure. In the first passage, a monkey was treated at 25 mg total dose. The drug dose was gradually increased in 12 successive passages over a period of 174 days and a dose of 150 mg (total dose) was administered in the 12th passage. Several isolates were cryopreserved in different passages to check the chloroquine sensitivity at intervals. The parent strain (W_1) of *P. knowlesi* has been found to be curative at 7.5 mg base/kg chloroquine x 3 days. The chloroquine sensitivity of isolates cryopreserved during 11th passage was determined at 10.0, 15.0 and 20.0 mg/kgx3 days. The results showed that the parasite was resistant to a dose of 10 mg/kgx3 as treated monkey recrudesced 11 days after end of treatment. The level of resistance was revalidated in two monkeys and stable resistant line could not be established.

ii) Selection by interrupted subcurative therapy

Attempts have also been made to select a chloroquine resistant strain of *P. knowlesi* by administering subcurative doses of chloroquine at interrupted intervals so as to allow constant drug exposure to the parasite. The first rhesus (Rh-I) was exposed to 5 doses of chloroquine ranging between 0.5-0.3 mg/kg during 8 days after which parasites were transferred to the naive monkey (Rh-II). Rhesus RH-II was exposed to 25 doses of chloroquine ranging between 0.2-0.3 mg/kg. The parasite has been subsequently passaged in four naive monkeys Rh III, Rh IV, Rh V and Rh VI as indicated in Figs. 3-8 and the subcurative chloroquine therapy was

continued (Table 38). The strain was maintained under constant drug pressure for nearly 14 months (Figs. 3-8). The periodic sensitivity tests performed periodically indicated no escalation of chloroquine curative dose of 7.5 mg/kg chloroquine base x 3 days.

B. Selection of Mefloquine resistant *P. knowlesi* in rhesus Monkey

Three rhesus monkeys No. 1, 2 and 3 were infected with *Plasmodium knowlesi* (W_1 strain) by inoculating $1x10^6$ parasitized RBC intravenously. The thick and thin blood smears stained with Giemsa stain were observed for recording parasitaemia. The three monkeys were treated with different doses of mefloquine (80, 40 and 20 mg/kgx3 doses) by oral route.

Monkey No. 1:

On day 3 of infection when the parasitaemia was approximately 0.7% a dose of 80 mg/kg mefloquine hyrochloride was administered for 3 consecutive days. The monkey was parasite negative after the second dose. The parasitaemia showed recrudescence 55 days after the third dose of mefloquine. The parasitaemia rose to 2.5 and 8.0% on day 58 and 60 respectively (Fig.9A). On day 60 the monkey was treated with 7.5 mg/kg chloroquine (base) orally for 3 successive days with a view to determine the sensitivity of the parasite to chloroquine. The monkey remained negative after chloroquine treatment till follow up of 40 days. The parent line resistant to 80 mg/kg dose of mefloquine has been cryopreserved for resistance reversal study.

Monkey No. 2:

When the initial parasitaemia was 0.5%; the monkey was treated orally with 40 mg/kg, mefloquine hydrochloride for 3 consecutive days. The parasitaemia became -ve after the second dose, but there was recrudescence on day 11 after the last dose of mefloquine. This monkey was again treated with 40 mg/kg mefloquine orally for 3 consecutive days and was cured (Fig. 9B).

Monkey No. 3:

The 3rd monkey with 0.3% parasitaemia, was treated with 20 mg/kg mefloquine hydrochloride orally for 3 consecutive days. The monkey was negative after the second dose. There was recrudescence on day 9 of the last dose of mefloquine. The monkey was again treated with 20 mg/kg mefloquine orally for 3 consecutive days. The monkey showed absence of parasitaemia after the second dose. On day 8 after the last dose, the monkey showed recrudescence. 20 mg/kg mefloquine was again administered orally for 3 consecutive days. The monkey was cured after the third dose of mefloquine but there was recrudescence and the parasitaemia reached 1.4% on day 14 after the last dose of mefloquine. The parasitized RBC were preserved in liquid nitrogen. The monkey was cured with 7.5 mg/kg chloroquine base x 3 day orally (Fig. 9C).

11. DRUG RESISTANT RODENT MALARIA STRAINS

(i) The following drug resistant lines of rodent malaria parasite *P. berghei* have been cryopreserved.

1. Chloroquine resistant strain (resistant upto 128 mg/kgx4 doses)
2. Mefloquine resistant strain (resistant upto 128 mg/kgx4 doses).

3. Quinine resistant strain (resistant upto 400 mg/kgx4 doses).

(ii) A multiple resistant strain of *P. yoelii nigeriensis* resistant to chloroquine (128 mg/kgx4), mefloquine (128 mg/kgx4) and quinine (400 mg/kgx4) has been cryopreserved.

12. STUDIES ON REVERSAL OF DRUG RESISTANCE

A large number of reports have appeared in literature during the last decade in which the chloroquine resistance of the cultured drug resistant isolates of *P. falciparum* had been claimed to be reversible *in vitro* by certain agents/compounds designated as reversal agents/resistance modulators/MRD modifiers. In presence of resistance reversal agents, a much lower dose of chloroquine is required to kill the resistant *P. falciparum* in culture. So far, very few drug resistance reversal studies have been carried out in the *in vivo* malaria models. But the published data do not prove conclusively that available resistance reversal agents would be potentially safe clinically and effective. Efforts were, therefore, continued to establish chloroquine/mefloquine resistant simian malaria secondary screening models to evaluate these claims and also to complete preclinical studies on a few selected reversal agents, which could be identified as potential candidate compounds for clinical trials.

A. Drug resistance reversal studies against multi-resistant *P. yoelii nigeriensis*

This strain is resistant to chloroquine (128 mg/kg x 4), mefloquine (128 mg/kg x 4) and also quinine (400 mg/kg x 4) and it is 100% lethal for Swiss mice.

VERAPAMIL

Verapamil which is a calcium channel blocker has been evaluated for chloroquine resistant reversal activity against multiresistant *P. yoelii nigeriensis*. Two drug administration schedules from day 0-3 and day 3-6 post-infection were used.

(i) **Day 0-3 treatment**

Chloroquine alone was given at 8 mg/kg dose, verapamil at 25 mg/kg. Besides a combination of verapamil 10 and 25 mg/kg with 8 mg/kg dose of chloroquine was tested (Table 39). Mean survival time (MST) of verapamil and chloroquine combination was slightly extended (12.25-12.63 days) in comparison to MST of 10.75 days observed in chloroquine control group. Extension of MST was observed only at higher doses of verapamil (10 and 25 mg/kg) and no extension of MST was observed with lower dose of verapamil (0.5 and 1.0 mg/kg).

(ii) **Day 3-6 treatment**

In this second group, the drug administration schedule was from day 3-6 post-infection. Chloroquine treated group of mice showed MST 21.14 days whereas different doses of verapamil with 8 mg/kg chloroquine showed mean survival time ranging from 15.17, 23.67, 24.60 to 25.57 days and the increase in MST was directly related to the increasing dose of verapamil from 5-25 mg/kg (Table 40). The study shows a limited reversal effect of verapamil when given with chloroquine. It may be pointed out that the number of animals surviving with combination of verapamil and chloroquine has not been consistent in different experiments.

NIFEDIPINE

This drug was also tested in combination with chloroquine against multi-drug resistant *P. yoelii nigeriensis* using 3-7 day post-infection treatment schedule. Before drug treatment the parasitaemia was 0.5%. In groups given nifedipine + 8 mg/kg chloroquine, the maximum survival time was 24.7 and 24.8 days in comparison to chloroquine alone which gave 21.14 days (Table 41). In conclusion the nifedipine has provided marginal extension of MST, specially at the high dose.

EVALUATION OF WR 238605 FOR CHLOROQUINE RESISTANCE REVERSAL ACTION

For resistance reversal studies with WR 238605, chloroquine resistant strain of *P. yoelii nigeriensis* was used. Chloroquine treatment (4.0 and 8.0 mg/kg x 4 days) resulted in MST of 12.8±4.2 and 17.8±9.4 days respectively, while chloroquine at 4.0 and 8.0 mg/kg when given together with 0.5 mg/kg of WR 238605, resulted in only slight extension of MST from 12.8±4.2 to 14.4±5.9 at 4.0 mg chloroquine dose, and from 17.8±9.4 days to 19.4±9.0 days at 8.0 mg chloroquine dose. Administration of WR 238605 (0.5 mg/kg) with chloroquine (4.0 or 8.0 mg/kg) extended the MST by nearly 2 days at both the dose levels of chloroquine used in the study (Table 42).

The marginal extension of MST when WR 238605 is administered with chloroquine suggests some additive effect of the drug combination specially when both the drugs are blood schizontocides.

EVALUATION OF WR 238605 FOR MEFLOQUINE RESISTANCE REVERSAL ACTION

Day 0-3 treatments

Resistance reversal effect of WR 238605 (0.5 mg/kg dose) alone and in combination with various doses of mefloquine (1.0, 2.0, 4.0 and 8.0 mg/kg x 4 days) was evaluated using multi-resistant *P. yoelii nigeriensis*. This rodent parasite is resistant to mefloquine at 128 mg/kg x 4 days schedule. WR 238605 (0.5 mg/kg) alone did not extend the mean survival time of the mice which was 6.2 days compared to 5.8 days in control group (Table 43). Mefloquine alone (1.0-8.0 mg/kg doses) produced gradual increase of MST from 6.6 days to 15.0 days corresponding to increasing dose levels of mefloquine. When mefloquine doses (1.0, 2.0, 4.0 and 8.0 mg/kg) were given together with fixed dose of WR 238605 (0.5 mg/kg) there was no increase in MST which varied from 6.6, 10.0, 11.0 to

13.2 days respectively corresponding with the increasing dose level of mefloquine. The study shows no significant resistance reversal effect of WR 238605 against mefloquine resistant strain of parasite.

Day 3-6 Treatment

Additional studies using WR 238605 in therapeutic schedule i.e. day 3-6 post-infection using the same multi-resistant strain also shows no significant resistant reversal effect since the mean survival time of the mefloquine alone at different dose was 26.0, 31.50 and 37.66 days respectively which were longer as compared to corresponding combination treatment groups (WR 238605 + mefloquine), the MST being 21.5, 35.33 and 29.33 days). Mefloquine being a long acting compound provides prolonged suppression of blood parasitaemia. Slightly better suppression of parasitaemia on day 7 in WR 238605 + mefloquine groups, as compared to mefloquine alone groups, suggests some transient additive action of the two compounds (Table 44).

EVALUATION OF QUINIDINE FOR CHLOROQUINE RESISTANCE REVERSAL EFFECT

Day 3 to 6 treatment

Quinidine which is known to be effective against chloroquine resistant *P. falciparum*, was evaluated for possible additive antimalarial or resistance reversal effect in combination with chloroquine using multiresistant *P. yoelii nigeriensis* rodent strain. Results of the experiments in which therapeutic treatment with quinidine alone, chloroquine alone and combination of both quinidine with chloroquine were given from day 3-6 post-infection when the initial parasitaemia was in range of 2.5% are given in Table 45).

Analysis of results on day 10 post-treatment suggests a significant decrease of parasitaemia in group given quinidine and chloroquine combination (0.33 ± 0.08) in comparison to quinidine alone (5.95 ± 0.15) and chloroquine alone (5.2 ± 1.5). However, overall assessment of the data on mean survival time basis show that the

chloroquine treated group of mice survived for 11.55 days, quinidine alone group showed 11.83 days and chloroquine and quinidine groups survived for 24.16±9.08 to 27.0 days, suggesting the extension of mean survival time in the combination group. Overall data suggest that quinidine in combination with chloroquine exerts possibly resistance reversal effect since 4 out of 6 mice survived in quinidine + chloroquine combination groups but there was no survival in quinidine or chloroquine treated groups (Table 45)..

B. Resistance reversal studies with mosquito transmissible *P. yoelii nigeriensis* (N-67) in Swiss mice

a) Selection of resistant strains

Four drug resistant strains showing resistance to chloroquine (128 mg/kg), mefloquine (128 mg/kg), halofantrine (128 mg/kg) and pyrimethamine (48 mg/kg) were selected after exposing the parent drug sensitive parasites to interrupted subcurative therapy with the respective antimalarials (Table 46). The stability of resistance was confirmed after transmission through the vector *An. stephensi*. These strains have been used for resistance reversal studies using i) Verapamil, ii) Amitryptline and (iii) Cyproheptadine.

b) Resistant reversal studies with chloroquine resistant strain

Combination of chloroquine (16 mg/kg) and verapamil (50 mg/kg) showed marked reduction in parasitaemia on day 4 compared to the chloroquine alone or verapamil alone treated groups. The combination has only transient suppressive effect observed one day after the last dose, while there was no significant difference in the parasitaemia in the combination and chloroquine alone treated groups after day 7 (Table 47).. Antidepressant drug amitryptline in combination with chloroquine showed significant suppression of parasitaemia on day 4 and 7 compared to the corresponding controls, showing a transient suppressive efficacy of the combination (Table 47).

Resistance reversal studies with combination of cyproheptadine and chloroquine showed that the animals treated with combination of chloroquine (16 mg/kg) and cyproheptadine (10 mg/kg) were completely protected upto day 28 observation (Table 47).

c) Resistance reversal studies with mefloquine resistant strain

Studies using cyproheptadine in combination with mefloquine against mefloquine resistant strain showed significant activity of the combination. 70% of the combination treated animals did not develop any parasitaemia during the observation period while only transient low level parasitaemia was observed in the remaining 30% animals (Table 48.). The resistance reversal potential of cyproheptadine warrants further evaluation in the primate malaria model.

Combination of mefloquine 8 mg/kg with amitryptline (50 mg/kg) showed significant suppression of parasitaemia on day 4 and 7 while mefloquine plus verapamil combination produced only transient suppression of parasitaemia compared to the corresponding controls (Table 48).

d) Resistance reversal studies with Halofantrine resistant strain

Halofantrine (4 mg/kg) in combination with cyproheptadine (10 mg/kg) protected all the treated mice during observation period of 28 days while partial reversal effect was observed with verapamil or amitryptline combinations (Table 49).

e) Resistance reversal studies with Pyrimethamine resistant strain

Combination of pyrimethamine 4 mg/kg with cyproheptadine (10 mg/kg) or with amitryptline (50 mg/kg) did not show any significant variation of parasitaemia from the group treated with pyrimethamine alone (Table 50).

C. Resistance reversal studies in simian malaria model/*P. knowlesi* rhesus monkey

Plasmodium knowlesi infection in rhesus monkey has been found to possess

innate resistance to mefloquine and the parasite has been found to show recrudescene even after 80 mg/kg x 3 days treatment. This simian model has been used for evaluating the resistance reversal efficacy of amitryptline and cyproheptadine.

Amitryptline

Two monkeys were inoculated with $1x10^6$ *P. knowlesi* blood stage parasites and when parasitaemia reached between 2-3%, the monkeys were treated with 20 mg/kg mefloquine x3 days plus 20 mg/kg amitryptiline x 5 days. The parasite clearance was observed in 48 hours; however, both the monkeys showed recrudescene on day 11 and 13 after the last dose of mefloquine (Fig. 10) indicating no resistance reversal action of amitryptiline. Mefloquine (20 mg/kgx3 days) alone showed recrudescence on day 9 (Fig. 11).

Cyproheptadine

P. knowlesi infected monkeys at (0.3-3.7 %) were administered 20 mg/kg mefloquine x 3 days plus cyproheptadine (0.6-10 mg/kg) x 5 days (Table 51). The parasitaemia clearance was recorded within 48-72 hrs. Subsequent observation upto day 60 did not show any recrudescence in any of the monkeys treated with mefloquine plus 10 or 5 mg/kg cyproheptadine while partial protection was recorded with lower doses of cyproheptadine. One monkey was treated with 10 mg/kgx5 day cyproheptadine alone, and this dose cleared the parasitaemia in 72 hrs, though there was recrudescence after 3 days (Table 51).

D. Studies on mechanism of resistance reversal

Several models and working hypothesis for the mechanism of resistance and resistance reversal have been proposed. In this context the most recent findings that the cytochrome P-450 (Cyt. P-450) dependent hydroxylase activities are higher in CQ resistant than in sensitive strain are of significance. In eukaryotic cells these mono-oxygenase systems of which cyt. P-450 is the terminal oxidase, are responsible for the metabolism of a wide variety of structurally unrelated

xenobiotics, including antimalarial drugs and endogenous compounds. In the present investigation we will characterize the cyt. P-450 system in malarial parasite. The method for biochemical localization of cyt. P-450 in the microsomal fraction of *P. knowlesi* has been standardized and the cyt. P-450 has been partially purified. It is presumed that the drug resistant parasites would show increased level of specific activity of cyt. P-450 enzyme in comparison to the sensitive counterpart. Further, it is believed that the resistance reversal agents such as verapamil and nifedipin etc. would tend to down regulate the P-450 levels of the drug resistant *Plasmodia*. It is proposed to test this hypothesis in a multidrug resistant rodent malaria (*P. yoelii*) model which shows high level of resistance to chloroquine, mefloquine and quinine and the reversal agents would be administered for 4-7 days.

13. *IN VITRO* CULTIVATION AND BIOASSAY FOR ANTIMALARIALS

A. *In vitro* cultivation of simian malaria parasite (*P. knowlesi*)

Studies have been carried out to standardize culture conditions for short as well as long term *in vitro* maintenance of simian malaria parasite *P. knowlesi*. The parasites were maintained in RPMI 1640 medium supplemented with 10% normal monkey serum using candle jar technique. Infected blood at 2% parasitaemia was collected aseptically in citrate saline. Infected blood was washed with incomplete medium and finally 6% haematocrit was prepared in complete medium and dispensed 3 ml in petri dishes or glass vials. The medium was changed at every 24 hours and thin smears were prepared to monitor the growth of the parasites.

Preparation of media

RPMI-1640	10.4 gm
HEPES buffer	5.94 gm
Gentamycin	40.0 mg
Distilled water	900 ml

The contents were dissolved and adjusted to 960 ml, sterilized by filtering the medium through 0.22 μm millipore filter and dispensed in 100 ml volumes in sterile screw cap bottles for storage.

Sodium bicarbonate solution (5%)

$NaHCO_3$ anhydrous	5.0 gm
Distilled water	100 ml

Dissolved and sterilized by millipore filtration and stored in screw-cap tubes in 5 ml aliquotes.

Normal monkeys serum (NMS)

Fresh blood was collected from normal monkeys by venous puncture and allowed to clot at room temperature for 30 minutes. After storage at 0°C overnight the serum was collected and dispensed into sterile tubes. Serum was inactivated at 56°C for 30 minutes.

Complete medium

4.2 ml of 5% $NaHCO_3$ was added to 95.8 ml of the incomplete medium. Finally 10 ml NMS was mixed with 90 ml of the above medium.

The growth of *P. knowlesi* was good in medium supplemented with 10% NMS. Nearly 60-70% of the parasite matured into schizont stage in 24 hours. Invasion into new erythrocytes was observed for five-six cycles. *P. cynomolgi* cultured in medium supplemented with 10% NMS showed the parasite maturation from ring to schizont in 48 hours but invasion rate was very low. However, in medium supplemented with 20% NMS the growth of parasite was better and parasites were maintained upto day 15.

B. OPTIMIZATION OF CULTURE CONDITIONS FOR P.KNOWLESI

To optimize culture conditions parasites were obtained either from continuous cultures or from the infected rhesus monkey and diluted to obtain appropriate parasitaemia.

a. Growth of P.knowlesi in different culture vessels:

Four different polystyrene culture vessles namely 96 well plate (96 W-P) (Tarsons), 24 well plate (24W-P) (corning), 35 mm petri dish (35-PD) (Tarsons) and 25 cm^2 canted neck flask (25-F) (Tarsons) were used to find out the vessel supporting optimum growth to be used for subsequent experiments. According to the surface area of the respective vessel, cultures in different volumes were added to maintain a constant media height of 0.3 cm and thereby a uniform settlement of erythrocytes at the bottom of each vessel.

The results in Table 52 show that growth of P.knowlesi was more or less similar in all the vessels except in 96 W-P which reached an average parasitaemia of 2.5% at 72 hours as compared to 3.4%, 3.5% and 3.8% in 24W-P, 35-PD and 25-F respectively. Consequently, the multiplication rate was 5.0, 6.8, 7.0 and 7.6 respectively at 72 hours. For subsequent optimization studies, 24W-P was used throughout.

b. Growth of P.knowlesi at different haematocrits:

Growth of P.knowelsi at 3,6, 12 and 18% haematocrits with an initial parasitaemia of 0.6% is shown in Table 53. Though the effect of haematocrit was not pronounced significantly at 24 hour, it became obvious at 72 hour that higher haematocrits of

6% and above are suitable for the growth of P.knowlesi. At 72 hours, the parasitaemia at 3,6, 12 and 18% haematocrits was 2.7%, 4.9%, 5.7% and 4.5% respectively. A maximum multiplication rate of 9.5 was observed in 12% haematocrit at 72 hours followed by 8.2, 7.5 and 4.5 in 6% 18% and 3% haematocrit respectively.

c. Frequency of media renewal versus growth of P.knowlesi:

Table 54 shows the growth of P.knowlesi with respect to media renewal at every 12 hour and every 24 hour interval. The parasite grew in both the groups with insignificant difference. In once-in-24 hour renewal group, the parasite grew from 1.2% to 1.9% , 3.6%, 5.3% and 5.3% at 24, 48, 72 and 96 hours respectively In once-in-12-hour group, it multiplied to 2.1%, 4.3% , 5.1% and 5.9% in the respective hours.

d. Growth of P.knowlesi in various concentrations of rhesus serum:

P.knowlesi was cultured in presence of 10%, 20%, 50% and 100% of rhesus monkey serum (MS); the results are presented in Table 55.

Growth of P.knowlesi was directly proportional to the percentage of pooled MS supplemented in medium RPMI 1640 upto a concentration of 50%. After 72 hours incubation the peak parasitaemia of 5.5%, 11.8% and 13.3% was recorded in presence of 10% 20% and 50% serum respectively. Growth in 100% serum was almost as low as in 10% serum reaching a peak parasitaemia of 5.4% at 48 hour. In the cultures maintained without serum, parasitaemia declined down to zero at 72 hours(Table 55). Similar results were

obtained on growth of P.knowlesi in presence of various percentage of O(+) pooled human serum .

e. Comparison of growth of P.knowlesi in erythrocytes stored for various intervals:

The suitability of rheus erythrocytes stored at 4°C for 2,4,15, 30 and 40 days at 50% haematocrit in complete RPMI medium was evaluated for the growth of P.knowlesi and results are presented in Table 56. To increase the probability of merozoites to encounter experimental erythrocytes, parasitaemia as high as 8-10% from continuous cultures was diluted down to about 1% with experimental erythrocytes.

The results show a gradual decrease in the erythrocyte suitability with time of storage, rendering them totally unsuitable for growth of P.knowlesi above 15 days of storage. The parasitaemia in erythrocytes stored for 2,4 and 15 days gradually increased to 10%, 7% and 3.3% respectively at 72 hours as compared to an increase of 9.7% in fresh erythrocytes. Accordingly, the multiplication rate at 72 hour based on initial parasitaemia were 8.3, 7.8 and 2.8 against 8.1 in fresh cells. The parasitaemia in erythrocytes stored for 30 days increased slightly from 1.1% upto 1.4% at 48 hours. Erythrocytes stored for 40 days supported no growth of P.knowlesi.

f. Growth of P.knowlesi in erythrocytes stored at 4°C and 37°C.

Growth of P.knowlesi was compared in erythrocytes stored at 4°C and 37°C for 2,4 and 15 days. To increase the probability

of merozoites to encounter experimental erythrocytes, parasitaemia as high as 8-10% from continuous cultures were diluted down to about 1% with experimental erythrocytes. Table 57 shows that rhesus erythrocytes stored at 37°C lose their suitability for growth of P.knowlesi. Erythrocytes stored at 37°C for as short as two days could increase parasitaemia to 5.8% at 72 hours as compared to 9.7% and 10% respectively in fresh erythrocytes and erythrocytes stored at 4°C. Accordingly the multiplication rate was 8.1, 8.3 and 4.8 fold after addition of fresh erythrocytes, cells at 4°C and cells at 37°C for 2 days respectively.

Addition of erythrocytes stored for 4 days at 4°C and 37°C increased parasitaemia to 5% and 2.4% respectively at 48 hours. Storage of erythrocytes for 15 days at 4°C supported a gradual increase in parasitaemia to 3.3% at 72 hours whereas erythrocytes at 37°C supported a peak parasitaemia of 2.9% at 48 hours.

C. CONTINUOUS CULTIVATION OF P.KNOWLESI:

The observations on continuous in vitro cultivation of P. knowlesi upto a period of 35 days are represented in Table 58. The culture line was initiated with parasites obtained from an experimentally infected rhesus monkey at a parasitaemia of 1.4% ring stage parasites. Two days later, the parasitaemia increased by 1.3 fold reaching a level of 1.8% when the first addition of fresh rhesus erythrocytres was made. Multiplication rate improved after the initial two dilutions with a maximum of 4.2 fold increase between 21st and 23rd day. The parasitaemia ranged from 1.8% to 8.3% before dilutions in the 35 day period. Daily multiplication

rate during the 34 days of continuous cultivation averaged to 1.03 fold. The parasite number increased by a cumulative multiplication rate of 111061.7 fold on day 34 when the culture had undergone a cumulative dilution of 157464.

The presence of only rings in the first two days indicates the initial synchorny in vitro which was later lost.

D. STANDARDIZATION OF IN VITRO SCHIZONT MATURATION OF P.KNOWELSI:

As P.knowlesi maintains high natural synchrony during erythrocytic schizogony in vivo, parasites for standardization as well as for drug sensitivity assay were obtained from experimentally infected monkeys.

a. P.knowlesi : Time course of in vitro maturation:

Table 59 and figure represent differential count of naturally synchronous intraerythrocytes P.knowlesi during 34 hour duration. Early ring stage parasites at 0.93% parasitaemia and at 6% haematocrit in 200ul of C-RPMI were added to different wells and incubated in candle jar at 37°C. Smears from triplicate wells were prepared sequentially at different times intervals. The results in Table 59 show the formation of a few two nucleated schizonts as early as 14 hours. Multinucleated schizonts appeared at 16 hours. There was a gradual increase in the number of multinucleated schizonts reaching a maximum of 189 out of 200 parasites at 25th hour. Reinvasion of uninfected erythrocytes started from 26th hour which gradually increased to 196 per 200 parasites at 34 hours with a gradual reduction of schizonts which declined to 4 per 200 parasites at 34 hour. Apparently, the parasitaemia showed no major change

up to 25 hours but gradually started increasing to a maximum of 3.03% at 34 hours which is clear in Table-12. Thus, the ideal time for harvesting the DSA plate for P.knowlesi was determined to be between 22 and 25 hours.

b. P.knowlesi : Haematocrit verses schizont maturation:

Haematocrits of 3, 6, 12 and 18% in 200 u l of medium were used to monitor the maturation of P.knowlesi in a 22 hour period. Schizont maturation was higher with decreasing haematocrits (Table 60). The average number of schizonts per 200 parasites at 3,6, 12 and 18% haematocrits was 192, 165, 114 and 85 respectively. At 18% haematocrit, 30 out of 200 parasites could not even mature to trophozoites.

c. P.knowlesi : Volume of media versus schizont maturation

Ring stage parasites (0.9%) at 6% haematocrit in 50ul 100ul, 200ul and 300ul volumes were added to different wells and the schizont maturation was monitored at 22 hours. The results in Table 61 show more or less similar maturation pattern except in 300ul of media. The average schizont maturation of 182, 185 and 172 and 138 per 200 parasites was recorded in 50ul, 100ul, 200ul and 300ul of media respectively.

d. P.knowlesi: Initial parasitaemia versus schizont maturation:

Ring stage parasites at initial parasitaemia of 7%, 3.9%, 1% and 0.3% and at 6% haematocrit in 200ul of medium were incubated for 22 hours to monitor schizont maturation. Schizont maturation was inversely proportional to the initial parasitaemia giving average maturation of 32, 95, 147 and 187 schizonts per 200 parasites res-

pectively (Table 62).

E. STANDARDIZATION OF IN VITRO SCHIZONT MATURATION OF P.FALCIPARUM

Asynchronous P.falciparum cultures were synchronized to uniform ring stage parasite with 5% aqueous D-sorbitol and used for standardization as well as for drug sensitivity assay.

P.falciparum: Time course of in vitro schizont maturation

Differential count of intraerythrocytic stages of P.falciparum during 52 hour duration is shown in Table 63 and accompanying figure. The proportion of early: late rings obtained after sorbital synchronization was 148:52 out of 200 parasites. Smears in triplicate were made at different times as explained previously.

Preschizonts and schizonts started appearing from 16th hour onwards gradually increasing to a maximum of 34:90 of pre-schizont ratio at 30 hours. At 32 hours, reinvasion of uninfected red cells started giving an average count of 22 early rings per 200 parasites, which gradually increased at the expense of schizonts to a maximum of 152 per 200 parasites at 52 hours. Consequently, the parasitaemia started increasing from 32 hours onwards reaching a maximum of 3.53% at 52 hours (Table 63). Thus, the ideal time for harvesting the DSA plate for sorbitol synchronized P.falciparum was determined to be between 26 and 30 hours provided a higher proportion of early rings is available as in the present case.

F. EFFECT OF INITIAL PARASITAEMIA ON MINIMUM INHIBITORY CONCENTRATION:

(a) MIC against P.knowlesi:

THe MIC of chloroquine in two fold increasing concentration was tested for schizont maturation against P.knowlesi. The results showed that MIC is directly proportional to the parasitaemia level used for the test. The MIC value obtained with 11%, 1.6% and 0.25% initial parasitaemia was 125 ng/ml, 31.3 ng/ml and 15.6 ng/ml respectively (Fig. 12A).

(b) MIC against P.falciparum:

Chloroquine in two fold increasing concentrations ranging from 19.5 ng/ml to 5000 ng/ml was tested. The MIC at highest parasitaemia of 4.7% was 625 ng/ml but the value was identical (312.5 ng/ml) for 0.9% and 0.12% initial parasitaemia (Fig. 12B)

G. DRUG SENSITIVITY ASSAY OF ANTIMALARIALS AGAINST P.KNOWLESI

Several standard antimalarialsnamely chloroquine, mefloquine, halofantrine, quinine, pyronaridine and WR 238605 were tested in vitro against P.knowlesi. The tests were carried out with a view to establish base line values for these drugs against this new model with an initial parasitaemia of 0.5 to 1% and results are presented in Fig. 13 (A-F).

(a) Chloroquine:

Chloroquine concentrations ranging from 0.78 to 100 ng/ml were tested. The MIC of chloroquine against P.knowlesi was found

to be 50 ng/ml. Inhibition of 5.7%, 22.4% and 99.6% was found at concentration 6.25, 12.5 and 25 ng/ml respectively. No inhibition was observed at 3.13 ng/ml and lower concentrations (Fig.13A).

b. Mefloquine:

Mefloquine at a concentration range of 39 ng/ml of 5000 ng/ml in two fold increase was tested. The MIC of mefloquine against P.knowlesi was found to be 625 ng/ml. Below this concentration, 22.6% and 85.8% inhibition was recorded in presence of 156.3 ng/ml and 312.5 ng/ml mefloquine respectively. Still lower drug concentration did not produce inhibition of maturation of schizonts (Fig. 13 B).

c. Halofantrine;

A concentration range of 0.195 to 100 ng/ml in two fold increase was tested. No inhibition was found upto the concentration of 0.78 ng/ml. Inhibition of 14.6% , 32.6% and 85.4% was found at concentrations 1.56, 3.13 and 6.25 ng/ml respectively. The MIC of halofantrine against P.knowlesi was found to be 12.5 ng/ml (Fig. 13 C).

d. Quinine:

Quinine at concentrations ranging from 7.8 ng/ml to 1000ng/ml in two fold increase was tested. No inhibition was found up to 250 ng/ml. 500 ng/ml inhibited 24.6%. The MIC of quinine against P.knowlesi was found to be 1000 ng/ml (Fig. 13D).

e. **Pyronaridine:**

Pyronaridine at a concentration range of 0.78 ng/ml to 100ng/ml was tested. No inhibition was found up to 3.313 ng/ml of pyronaridine. Inhibitions of 31.3%, 52.3% and 86.5% were observed at 6.25, 12.5 and 25 ng/ml concentrations respectively. The MIC of pyronaridine against P.knowlesi was 50ng/ml (Fig.13E).

f. **WR 238605:**

A concentration range of 0.032 ng/ml to 2500 ng/ml in five fold increase was tested. No inhibition of schizont maturation was observed with this compound against P.knowlesi upto the drug concentration used in this study (Fig.13F).

A comparative assessment of the MIC values obtained for these antimalarials against P.knowlesi and P.falciparum is given below.

Drugs	**Minimum inhibitory concentration (ng/ml)**	
	P.knowlesi	P. falciparum
Chloroquine	50	200
Mefloquine	625	200
Halofantrine	12.5	1.56
Quinine	1000	625
Pyronaridine	50	62.5

H. Assessment of parasite growth *in vitro* by use of radiolabelled precursors

Different radiolabelled nucleotides and aminoacids have been used to measure parasite growth and study the inhibitory effects of drugs on the growth of the parasite. Optimum concentration of radiolabelled precursors and drug dilutions were added to the micro-cultures and the plates were further incubated for 18 hours. After incubation, the labelled parasites were harvested into the glass fiber filters using glass distilled water and an automated multiple sample harvester. The filter paper discs were added to 10 ml of scintillation fluid and counts recorded in a liquid scintillation counter. Results were expressed as disintegration per minute (DPM).

Comparison of uptake of different labelled precursors

Comparative uptake of ^{3}H labelled thymidine, leucine, isoleucine and hypoxanthine was determined during the growth of *P. knowlesi in vitro*. Labelled precursors (0.5 μCi) were added into the culture wells at 0-3 hr and plates were further incubated for 18 hours at 37°C in candle jar. After incubation cells were harvested in cell harvester. The filter paper disc was dried and placed in scintillation vial and counts recorded in scintillation counter. Results showed that ^{3}H thymidine, leucine and isoleucine incorporation was very low as compared to ^{3}H hypoxanthine uptake (Table **64**). Hence hypoxanthine was selected as the most suitable radiolabelled compound for *in vitro* drug assay studies. Webster and others (1981) have demonstrated that hypoxanthine is the major purine base utilized by the malaria parasite for synthesis of adenosine and guanine nucleotides and nucleic acids. The radio activity measured represents primarily ^{3}H hypoxanthine incorporation into the parasite. Background ^{3}H hypoxanthine incorporation by uninfected RBC's was low since these cells synthesize neither RNA nor DNA.

Standardization of optimum concentration of ^{3}H hypoxanthine for drug assay studies

P. knowlesi synchronized at ring stage was cultured in 96 well culture plates and radioactive counts after addition of 0.125, 0.25 and 0.5 μCi ^{3}H hypoxanthine were recorded after 18 hours incubation at 37°C in candle jar. Results showed that significantly high uptake of hypoxanthine was recorded with 0.5 uCi/well at parasitaemia levels rainging between 1-11% and ws found to be optimum for use in monitoring the growth *in vitro*. The uptake of hypoxanthine by uninfected cells was very insignificant (Table 65). Microscopic observations of Giemsa stained smears from cultures incubated under similar conditions (except addition of ^{3}H hypoxanthine) showed that most of the ring stage parasites had matured into the trophozoites and schizonts.

Effect of duration of incubation on uptake of ^{3}H hypoxanthine

P. knowlesi synchronized at ring stage was cultured in 96 well micro-culture plates and incubated at 37°C in candle jar. ^{3}H hypoxanthine (0.5 uCi in 20 μl medium) was added to each micro-culture. Comparison was made of the quantitative uptake of labelled hypoxanthine after 4 hr and 24 hr incubation using variable percent parasitaemia and haematocrit. Results showed that uptake was very low during initial 4 hours (i.e. during the maturation of rings into early trophozoites), while significant incorporation was observed after 24 hours of culture i.e. during the period of schizont maturation both at high (9%) and low (1%) parasitaemia levels (Table 66).

Effect of parasite number and haematocrit on hypoxanthine incorporation

Comparison was made of the uptake of hypoxanthine at parasitaemia levels of 9, 3 and 1% and normal red blood cells (NRBC), as well as at different haematocrit viz. 6%, 3%, 1.5% and 0.75%. Synchronized *P. knowlesi* at ring stage were cultured in micro-culture plates. Micro-cultures were pulsed with 0.5

uCi ^{3}H hypoxanthine for 24 hours to re-record the radio isotope incorporation. Results in Table 67 show that incorporation of ^{3}H hypoxanthine was directly proportional to the increase in parasitaemia from 1 to 9%. At 6% haematocrit, the uptake was proportional to increase in parasitaemia from 1 to 3% since at high parasitaemia (9%) there was decline in the DPM values. A comparison of the results on parasitaemia versus haematocrit basis showed that the uptake of ^{3}H hypoxanthine at high parasitaemia (9%) was inversely proportional to haematocrit concentration i.e. there was increase in uptake with corresponding decline in the haematocrit. On the other hand, at low parasitaemia of 1%, this relationship was direct i.e. increase in haematocrit resulted in increase in uptake of radioactive precursors. At medium parasitaemia level (3%), the uptake of hypoxanthine was more or less identical with all haematocrit levels used in the study. In the uninfected cells the counts were very low and nearly identical at all the haematocrit levels.

***In vitro* antimalarial screening model: Evaluation of dose response of chloroquine using ^{3}H hypoxanthine incorporation**

Limited studies have been conducted to determine the dose response of chloroquine. *P. knowlesi* synchronized at ring stage with 6% parasitaemia and 1.5% haematocrit were incubated with different concentration of chloroquine (0.00015 μg/ml-10.0 μg/ml) in 96 well micro-culture plates and incubated at 37°C in candle jar. Micro-culture were pulsed with 0.5 μCi ^{3}H hypoxanthine after four hours and further incubated for 18 hours. Micro-culture plates were harvested after incubation. The filter paper discs were added to scintillation fluid and activity counted in scintillation counter. Data was analysed for determination of IC50/IC90 values and results are presented in Table 68.

I. *In vitro* testing for tissue schizontocidal action

A method is being standardized for primary screening of protective tissue schizontocides using *P. cynomolgi* exoerythrocytic stages cultured in rhesus hepatocytes. Assay was standardized using standard tissue schizontocidal drug primaquine. The drug was added 24 hrs after sporozoite invasion of cultures. Primaquine exerted tissue schizontocidal action against the primary EE stages of the parasite at concentrations as low as 0.1 μg/ml. Simultaneous experiments showed that chloroquine did not exert any parasiticidal effect even at concentrations of 5 μg/ml.

This assay will be useful for primary screening of tissue schizontocides and will go a long way to replace the costly *in vivo* rhesus monkey model for conducting large scale evaluation of potential tissue schizontocides. This study will also provide new leads for identification of the site of action of tissue schizontocides.

J. Standardization of *in vitro* antimalarial assays system using parasite LDH

The development of *in vitro* antimalarial screening of potential antimalarial as well as establishment of new assay systems to detect drug resistance character of the malaria parasite is receiving high priority in the collaborative programme. So far, the identification of resistance is generally done in the *in vitro* model by giving four doses of drug and recording the level of infection/% suppression of parasiaemia of the drug treated animals as compared to the untreated controls. The parasite like *P. yoelii nigeriensis* MDR strain can tolerate high level of antimalarials *in vivo* and has shown resistance to 128 mg/kg x 4 days chloroquine. 128 mg/kg x 4 days mefloquine and 400 mg/kg quinine x 4 days.

This MDR parasite is now being used to establish an *in vitro* system for detection of drug resistance based on possible inhibition of LDH activity of the parasite in presence of drugs.

Two assay systems have been initially investigated for detection of parasite LDH.

1. Reaction mixture containing Tris-Lactate Buffer (52 mM) B NAD (172 mM), NBT (0.24 mM), MTT (0.033 mM).

2. Reaction mixture containing Tris-Lactate Buffer (52 mM), APAD (172 mM), NBT (0.24 mM), MT (0.033 mM).

APAD cofactor containing the reaction mixture has shown a very high level of parasite activity even at 10-15% parasitaemia in comparison to the normal blood (control) which shows a very low level of activity.

Fig. 12 (infected blood versus normal blood) shows high sensitivity of APAD for LDH parasite quantitation using Sherman method.

The LDH detection system with APAD as co-factor can be developed to establish the *in vitro* system for antimalarial screening.

14. Viability of parasites versus duration of drug exposure

Drug dilution

A drug stock of 1 mg/ml was prepared in appropriate solvent as described earlier. It was further diluted in incomplete medium (I-RPMI) and added to 30 ml of complete medium to obtain final concentration equivalent to 1-50 times the MIC values of drug against *P. falciparum*.

Incubation of parasite with drug

P. falciparum from continuous culture were synchronized with 5% D-sorbitol and diluted to 0.5 to 1% initial parasitaemia and incubated in drug containing complete medium each at 6% haematocrit in 35 mm disposable petridishes. Parasite in drug free medium served as control. The petridishes were incubated at 37°C in a candle jar for varying periods ranging between 3 and 72 hours. When the exposure time was longer than 24 hours, media with drug at the appropriate concentrations was replaced daily.

Reincubation in drug free medium

At 3, 6, 12, 24, 36 and 48 hours, different petridishes were removed from candle jar and the supernatant media above the settled layer of red cells was discarded. The cells were transferred to a centrifuge tube and washed thrice with I-RPMI. The washed cells were resuspended in 2 ml of drug free complete medium. 200 ml aliquots of the washed cells were transferred to 10 wells of 96 well micotitre plates. The plates were incubated in a candle jar. Medium was replaced daily. Incubation was continued upto 144 hours.

Growth monitoring

Thin blood smears were made at 48, 96 and 144 hours post drug exposure from triplicate wells. Smears were fixed and stained with Giemsa stain. The parasites were counted per 4000 RBCs and recorded as percent parasitaemia.

A. Viability of *P. falciparum* post chloroquine exposure

Table 69 represents mean (n=3) differential parasite count at 48, 96 and 144 hours after exposure to chloroquine at 200, 1000 or 5000 ng/ml concentration for 3, 6, 12, 24, 36, 48, 60 or 72 hours. The initial parasitaemia was 0.91%.

Parasite cultures exposed to 200 ng/ml chloroquine for 3 to 48 hours remained viable as evidenced by the increase in parasitaemia or by the appearance of trophozoites and schizonts after incubation in drug free medium for 144 hours. The duration of exposure had a drastic inhibitory effect on the growth of parasites which was confirmed by the considerable decrease in the total parasitaemia as compared to the controls. Moreover, the inhibitory effect of drug exposed for longer durations up to 36 or 48 hours was reflected by the delayed appearance of the remaining parasites. Though there was no indication of growth at 48 hour after drug exposure for 36 or 48 hours, the viability of parasites was confirmed by the appearance of trophozoites and schizonts only at 96 or 144 hour respectively post drug exposure. In culture exposed for 60 or 72 hours, the drug caused a total parasiticidal effect and therefore, no indication of growth was recorded upto 144 hours of maintenance in drug free medium.

When cultures were exposed to higher concentration of 1000 or 5000 ng chloroquine per ml, the viability of parasites w as observed only up to the maximum exposure time of 36 hours. The growth pattern was similar with both the concentrations. The growth of parasites was considerably affected by the length of exposure to both the concentrations of chloroquine as compared to the control parasitaemia. After 36 hours of exposure to 1000 or 5000 ng/ml concentrations, no indications of growth was observed at 48 hours post exposure in drug free medium. However, 0.01% or trophozoites were observed at 96 hours post exposure in both the groups which further increased at 144 hour. A duration of at least 48 hours with both the higher concentrations was needed for the complete growth inhibition of parasites.

Thus the overall inhibitory effect after exposure to chloroquine on the *in vitro* growth of the remaining parasites of *P. falciparum* resulted in an inversely proportional relationship with the duration and concentration of drug exposure.

B. Viability of *P. falciparum* post mefloquine exposure

Table 70 shows mean (n=3) differential parasite count at 48, 96 and 144 hours after exposure to mefloquine at concentrations of 200, 1000 or 5000 ng/ml for 3, 6, 12, 24, 36, 48, 60 or 72 hours. The initial parasitaemia was 0.91%.

Cultures of *P. falciparum*, synchronized at ring stage were exposed to 200 ng/ml mefloquine for 3 to 72 hours. A complete inhibitory effect was observed when the parasites were exposed for 24 hours or more since, there was neither an increase in parasitaemia nor any appearance of trophozoite or schizont stages of parasites in culture up to 144 hours of post exposure cultivation in drug free medium. On the other hand, the exposure of parasites to 200 ng/ml mefloquine for shorter durations viz., 3, 6 and 12 hours did not result in total loss of viability, as parasites could continue the *in vitro* growth when allowed to grow in drug free medium. However, the exposure of parasites to higher concentrations of 1000 ng/ml or 5000 ng/ml resulted in complete loss of parasite viability ever after 3 hours of drug exposure, as parasites failed to multiply or grow in drug free medium (Tabl 70).

15. *In vitro* assay for antimalarial activity in serum

Selection of monkeys by prebleed sampling

Rhesus monkeys of 3.5 to 6 kgs were selected and samples of blood were collected from each monkey by venous puncture using 2 ml disposable plastic syringes. Blood was transferred to 5 ml glass test tube and allowed to clot at room temperature for one hour. After overnight incubation at 4°C, the serum was separated by centrifugation at 2000 rpm for 15 minutes. The serum was sterilized through 0.45 µm millipore filter and assessed for suitability for parasite maturation *in vitro*. The monkeys showing more than 70% schizont maturation were selected for the study.

Serum collection

5 ml of blood was collected prior to drug administration and designated as "0" hour sample. After drug administration, 5 ml samples of blood was serially collected at 1/2, 1, 2, 4, 6, 12, 24, 30, 36, 48, 72, 96, 120 and 144 hours post drug treatment. The serum was separated and filtered through 0.45 µm The samples were stored frozen in 2 ml sterile screw cap plastic vials (Laxbro) until assessment of drug activity.

Assessment of drug activity in serum

Assessment of serum drug activity against *P. knowlesi* and *P. falciparum* was carried out using sterile 96 well flat bottomed plates. Ring stage parasites containing 0.5 to 1% parasitized RBCs were prepared in I-RPMI at 7.5% and 12% haematocrits separately and incubated in presence of different samples of serum collected above.

a. Persistence of serum drug activity

Persistence of serum drug activity was assessed by adding 25 µl of the serum samples in triplicate wells. To this 25 µl of parasites at 12% haematocrit was added. The plate was incubated at 37°C in a candle jar upto the time of schizont maturation and then the test was harvested. The percent inhibition of

maturation was calculated as described for DSA. The respective '0' hour samples served as controls.

b. Activity kinetics of drug in serum

Activity kinetics of drug in serum was determined by calculating the maximum inhibitory dilution (MID) of each serum which showed 100% inhibition of schizont maturation. To determine this, 40 µl of each serum sample which showed 100% inhibition of schizont maturation was further serially diluted with NMS in two fold dilution upto a maximum of 1/320 in 96 well plates. 20 µl of each dilution was transferred to the adjacent well to serve as duplicate. The first well received 20 µls of undiluted drug sera. After dilution, 80 µl of parasite suspension at 7.5% haematocrit was added to each well and incubated in a candle jar at 37°C. The drug free diluent serum served as controls. After incubation, the test was harvested and the MID values of each sample was determined.

The MID was plotted on a graph against time of sample collection to demonstrate the activity kinetics of drug in monkey serum.

Bioassay of drug equivalent in serum samples

Drug equivalents were calculated by extrapolating the MID values according to the method of Kotecka & Rieckmann (1995). This essentially relates the MID of a parasite isolate to the MIC of drug for that isolate and converts the results into drug concentration equivalents. Therefore, drug equivalents were calculated as follows :

$$\text{Drug Equivalent} = \text{MID} \times \text{MIC}$$

The calculated drug equivalents in serum were expressed in ng/ml.

A. *Ex vivo* bioassay of chloroquine

Ex vivo bioassay of chloroquine against *P. knowlesi*

Table 71 shows the results on percentage inhibition of schizont maturation against *P. knowlesi* and the calculated choloroquine equivalents (CHL-Eq) based on the MID values shown in Fig. 11 in serum samples collected between 1 and 144 hours post administration of single dose of 10, 20 or 30 mg chloroquine base/kg to six rhesus monkeys.

i. Persistence of serum chloroquine activity

Serum chloroquine activity as assayed by inhibition of schizont maturation shows complete inhibition up to 144 hours after chloroquine treatment at 10 mg/kg and 30 mg/kg in monkeys 103 and 106 respectively. Similar activity was also recorded in samples from other monkeys (101, 102, 104 and 105) which however were collected upto 30-120 hours (Table 71).

ii. Activity kinetics of serum chloroquine

The activity kinetics of chloroquine in serum was assessed by determining the maximum inhibitory dilution (MID) of the samples. Fig. 14(A, B, C) shows the results on maximum inhibitory dilution of the same serum samples showing inhibition of schizont maturation of *P. knowlesi*. In serum samples from 10 mg/kg treated monkeys, complete inhibition was observed upto1:10 dilution in samples collected between 2-24 hours for monkey 101,

between 2-72 hours for monkey 102 and 1-96 hours for monkey 103. Three samples (12,24,36 hrs.) from monkey 102 and 4 samples (6,12,24 and 36 hours.) from monkey 103 also inhibited schizont maturation upto 1:20 dilution. After treatment of 20mg/kg dose (monkey 104), 2-36 hour samples inhibited schizont maturation upto 40 times dilution. Out of the two monkeys treated with 30 mg/kg, serum samples collected after 2,4,24 and 30 hours from monkey 105 showed complete inhibition up to a dilution of 1:40 while samples collected at 6 and 12 hours gave an MID of 80. Monkey 106 showed an MID of 20 for samples collected at 1, 120 and 144 hours while samples collected between 2 and 96 hours gave an MID of 40 except the 36 hour sample whose MID was 80.

iii. Concentration of chloroquine equivalents in serum samples

Chloroquine equivalents were calculated by multiplying MID with MIC of chloroquine against *P. knowlesi*. The CHL-Eqs calculated against *P. knowlesi* (Table 71)in the dosage group of 10mg/kg started with 313 ng/ml at 1 hour reaching a peak value of 626 ng/ml either at 6 hour (monkey 103) or at 12 hour (monkey 102) and declined at 48 hour to 313 ng/ml which gradually further declined to 157ng/ml at 120 hours. However, in monkey 101 which received the same dose, CHL-Eqs were calculated to be 313 ng/ml in serum samples collected from 2 to 24 hours and 157ng/ml in last sample collected at 30 hours. CHL-Eqs in monkey 104 treated with 20 mg/kg peaked at 2 hours showing, a level of to 1252 ng/ml which persisted at the same concentration upto the last sample collected at 36 hours. With 30 mg/kg dose, CHL-Eqs value was 1252 ng/ml at 2 hours reaching a peak between 6 and 12 hours to 2504 ng/ml and declined to 1252 ng/ml at 24 hours which persisted at the same level till the last sampling made at 30 hours in monkey 105. In monkey 106 of the same dosage group, sample collected at 1 hours gave a chloroquine equivalent of 625 ng/ml, then increasing to 1252 ng/ml in samples collected between 2 and 96 hours with a peak value of 2504 ng/ml at 36 hours. Samples at 120 hour and 144 hour showed value of 626 ng/ml.

B. *Ex-vivo* bioassay of halofantrine

Ex-vivo bioassay of halofantrine against *P. knowlesi*

Table 71 depicts the per cent inhibition of schizont maturation against *P. knowlesi* and the calculated halofantrine equivalents (HAL-Eq) based on the MID values shown in Fig. 15 in serum samples collected from 2 to 120 hours after treatment with single halofantrine dose of 10, 20 or 30 mg/kg in five rhesus monkeys.

i. Persistence of serum halofantrine activity

Serum samples from monkeys 301 and 302 which received 10 mg/kg dose completely inhibited schizont maturation up to 30 hours (last sampling made) and 48 hours respectively. The 72, 96 and 120 hours samples from monkey 302 inhibited parasite maturation by 69%, 28% and 17% respectively. All the serum samples from the monkeys (303, 304 and 305) which received higher doses of 20 ng/kg and 30 mg/kg dose could cause 100% inhibition i.e. up to 36 hour sample from monkey 303, upto 30 hour sample from monkey 304 and upto 120 hour sample from monkey 305 (Table 74).

ii. Activity kinetic of halofantrine in serum

The activity kinetics of halofantrine was assessed by determining the MID of the individual serum against schizont maturation of *P. knowlesi*. The results are shown in Fig. 15 (A, B, C). Monkey 301 and 302 received a dose of 10 mg of halofantrine per kg. All serum samples collected up to 30 hours from monkey 301 gave an MID of 10 while all sample collected up to 48 hours from monkey 302 gave an MID of only 5. The 72, 96 nd 120 hour samples of monkey 302 did not interfere with schizont maturation even at the lowest

dilution of 2 times. Samples of monkey 303 (20 mg/kg dose) gave an MID of 40 for 2, 4 and 6 hours samples, 10 for 12, 24 and 30 hour samples and 5 for 36 hour sample. On the other hand with 30 mg/kg dose, samples from monkey 304 collected at 4, 6 and 12 hours inhibited upto 80 times dilution while 24 and 30 hour samples up to 40 times dilution. The 2 hour sample gave an MID of only 20 times. The samples of other monkey (No. 305) at the same dosage group gave an MID of only 20 up to 48 hours sample. The 72, 96 and 120 hours samples gave a lower MID of 10, 5, and 2 respectively.

iii. Concentration of halofantrine equivalents in serum

The HAL-Eq calculated from the MID values showed that 125 ng/ml of HAL-Eq persisted constantly from 2 hours till the last sampling made at 30 hours in monkey 301 administered with 10 mg/kg dose. The other monkey (No: 302) which received the same dose showed a constant HAL-Eq values of 63 ng/ml in all the samples collected between 2 and 48 hours. With 20 mg/kg dose, monkey 303 started with a peak concentration of 500 ng/ml declined to 125 ng/ml at 12 hour and ended at 63 ng/ml at the last sampling made at 36 hours. At 30 mg/kg dose in one of the two monkeys (monkey 304), the HAL-Eq concentration peaked between 4 and 12 hours to 1000 ng/ml, declined to 500 ng/ml at 24 hours and remained same till the last sampling made at 30 hours. The other monkey, No.305, started with a peak concentration of 250 ng/ml at 2 hours which maintained the same level till 48 hours and then gradually waned to the lowest detectable limit of 25 ng/ml at 120 hours (Table 72).

16. *IN VITRO* METHOD FOR EVALUATING METHEMOGLOBIN TOXICITY OF 8-AMINOQUINOLINES

There is a major emphasis in the project on developing *in vitro* protocols for comparative evaluation of methemoglobin toxicity of potential 8-aminoquinoline agents. The protocol is being standardized using primaquine as the reference drug. For MetHb *in vitro* assay mastomys erythrocytes were incubated with varying concentrations of the drug for 90 minutes. Methemoglobin formed was recorded at 630 nm and percentage was calculated with reference to the total hemoglobin in the lysate.

Standard compound N_aNO_2 was also used as the reference to standardize the test, since it is known to convert Hb to MetHb in 15-20 minutes. The results show linear increase in MetHb formed at concentration between 10 to 1000 μm. The chloroquine used as reference negative drug did not produce appreciable MetHb, while reference 8-aminoquinoline drug primaquine produced MetHb. 3-4.5% at 10 μm, 8.0-11.6% at 100 μm and 23.8-29.6% at 1000 μm concentration. MetHb formed with 4-methyl primaquine at 100 μm was twice the values obtained with primaquine at the same concentration (Table 73).

17. PROPHYLACTIC STUDIES WITH RECOMBINANT IL-12 AGAINST SPOROZOITE INDUCED *P. CYNOMOLGI* B INFECTION IN RHESUS MONKEYS (SPONSORED BY U.S. NAVAL MEDICAL RESEARCH INSTITUTE)

Experimental Procedures

Course of *P. cynomolgi* infection in rhesus monkeys: Intravenous injection of *P. cynomolgi* sporozoites results in universal blood stage infection about 10 days later (range 8-12 days). *P. cynomolgi* is a relapsing malaria, similar to human vivax malaria. Relapses generally occur 10-15 days after clearance of blood-stage infection. In spleen intact animals, parasitaemia ranges from 3-8%. Parasitaemias are approximately twice as high in splenectomized animals. The infection in rhesus monkeys is generally self-limited and the monkeys exhibit no overt distress; they eat and drink normally at all levels of parasitaemia. Mortality does occur, generally in splenectomized animals with high parasitaemias. Analgesics are generally not required. Infected animals can be cured with chloroquine 5 mg/kg and primaquine 1 mg/kg for 7 days. Rhesus monkeys weigh approximately 5 kg.

(i) Determination of protective dose and schedule of IL-12 in prophylactic test

Five groups of 4 monkeys have been tested. The formulation was delivered subcutaneously in the nuchal region. rHu IL-12 was diluted in sterile 1% normal monkey serum in PBS (pH 7.2) to give required doses of rHu IL-12 in 1.0 ml.

Control monkeys were given 1.0 ml. 1% monkey serum in PBS (pH 7.2). rHU IL-12 was given to the following regimens:

Group	IL-12 dose	Treatment duration	No. of monkeys
1.	100 ng/kg	Day-2 to +10(alternate day)	4
2.	1 μg/kg	Day-2 to +10 (alternate day)	4
3.	10 μg/kg	Day-2 (Single dose)	4
4.	20 μg/kg	Day-2 and 0	2
5.	Control (vehicle)	Day-2 to +10 (alternate day)	4

Revalidation of IL-12 efficacy

1.	10 μg/kg	Day-2 (Single dose)
2.	Control (Vehicle)	Day-2 (Single dose)

IL-12 prophylactic efficacy was revalidated in an additional experiment consisting of one study group (3 monkeys) and one control group (1 monkey).

Background

Testing to date has proven rHu IL-12 safe in monkeys. The above doses were recommended by researchers of Hoffman-LaRoche who have performed several rHu IL-12 studies in both rhesus and *Siamiri scirurus* monkeys. In Siamiri monkeys the above doses were bioactive and safe, with no clinical abnormalities or serious toxicity. Hematologic and serum chemistry abnormalities included mild to moderate anemia and leukocytosis, hypoproteinemia, hypoalbuminemia, hypophosphatemia, and hypocalcemia. Their findings suggest that the above doses might be active and not cause serious adverse effect. Earlier work in mice at

Naval Medical Research Institute with higher IL-12 per weight doses did not show adverse effects.

Sporozoite challenge

On day 0 monkeys were injected in the mid-saphanous vein (using a 25 g needle and 3 ml syringe) with 10,000 sporozoites which had been dissected from the salivary glands of *Anopheles stephensi* mosquitoes fed on monkeys infected with *P. cynomolgi*. Beginning on day 7 after infection and continuing for 8 weeks, monkeys were bled from the marginal ear vein (approximately 20 μl by sterile lancet skin prick) to assess parasitaemia by Giemsa-stained blood smear. Smears were performed daily for the first 3 weeks and twice per week for an additional 5 weeks. Obtaining the blood sample does not require anesthesia and lasts less than one minute. Any discomfort felt by the animal is transitory. Prior to puncture the skin is swabbed with alcohol. All monkeys that developed parasitaemia were cured with chloroquine 5 mg/kg and primaquine 1 mg/kg for 7 days by oral catheter.

Human rIL-12 used in this study was produced in *E. coli* and provided by F. Hoffman-LaRoche, Nutely, NJ. On every other day dose was used because of the prolonged half-life of rHu IL-12 in monkeys (14 hours) compared to mouse IL-12 in mice (3 hours).

Results

The four vehicle control monkeys (Group 5) developed patent infection between day 10-12 post sporozoite challenge.1 Likewise four monkeys each in group 1 (100 ng/kg dose) and group 2 (1 μg/kg x dose) also developed patent infection between day 11-18 (Table 44). None of the 4 monkeys in Group 3 (10 μg/kg single dose) and 2 monkeys in Group 4 (20 μg/kg x 2 doses) developed patent infection up to observation period of 70 days post challenge. The results thus show prophylactic efficacy of r IL-12 at 10 μg/kg dose (Table 74).

In the revalidation experiment to confirm the protective dose, the vehicle control monkey became patent on day 10 while none of the 3 monkeys treated with 10

μg/kg dose developed patent infection till 70 days, post sporozoite inoculation and were protected (Table 75). The study shows very good prophylactic efficacy of Hu r-IL-12 against sporozoite induced *P. cynomolgi* B. Further studies on the length of prophylactic efficacy and the validation of minimum prophylactic dose would be useful.

Determination of cytokine levels after rHuIL-12 injection

As discussed above, the parasite killing effect of IL-12 appears to be mediated by IFN-r although this has not been assessed in the monkey model. To assess this relationship, we have determined serum levels of IFN-r and IL-12 and mRNA expression kinetics of IFN-r IL-6, IL-10, IL-12, IL-15 and TNF-α. Ten blood samples (approximately 3 ml each) were obtained from each monkey for determination of IFN-r levels; this included a baseline sample prior to rHu-iL-12 injection, alternate day samples from day 0 to day 10, and twice weekly samples from day 11 to day 25. Blood was drawn from the external saphenous vein of the leg using a 22 g needle and 5 ml syringe. Serum samples were separated and frozen for later transport to Dr. Ansari (CDC), Atlanta, for testing and quantitation of cytokines. The results showed that Group 3, administered 10 μg/kg single dose was the only group in which plasma r-Hu-IL-12 levels were above control limits. These levels peaked on day 0 and then dropped back to near baseline by day 4 (Fig. 17). Serum IFN-r levels in group 3 rose steadily after IL-12 administration, peaking on day 2 and returned to near baseline on day 11 (Fig.18).

There was significant increase in IFN-r, IL-6, IL-10, IL-12 and IL-15, mRNA expression in monkeys that received r-Hu-IL-12 and the results are shown in Tables 76 and 77.

Table-1 : Serial cyclic passages of sporozoite induced p.cynomolgi B in rhesus monkeys since March, 1993.

Sporozoite passage no.	Date of inoculation	Monkey No.	Sporozoite inoculum (i.v.)	Day of patency
87	6.3.93	7666	$1.44X10^6$	8
88	13.4.93	7679	$0.73X10^6$	9
89	20.5.93	7680	$1.64X10^6$	8
90	26.6.93	7775	$1.24X10^6$	8
91	5.8.93	7782	$0.70X0^6$	9
92	8.10.93	7827	$0.76X10^6$	9
93	1.12.93	7850	$1.14X10^6$	8
94	10.1.94	7831	$0.96X10^6$	8
95	14.2.94	7911	$1.54X10^6$	8
96	18.3.94	8018	$0.83X10^6$	9
97	29.4.94	8029	$1.14X10^6$	8
98	17.6.94	8084	$1.40X10^6$	8
99	5.8.94	8086	$0.72X10^6$	9
100	10.9.94	8179	$1.15X10^6$	8
101	28.10.94	8258	$0.86X10^6$	9
102	22.12.94	8240	$1.24X10^6$	8
103	10.2.95	8299	$0.75X10^6$	9
104	21.3.95	8307	$0.50X10^6$	9
105	26.5.95	8348	$0.30X10^6$	9
106	8.7.95	8405	$1.20X10^4$	10
107	25.7.95	8367	$0.22X10^6$	9
108	28.8.95	8426	$3.00X10^4$	10

Serial cyclic passages (continued)

(2)

109	5.10.95	8437	$1.40X10^4$	11
110	7.12.95	8467	$1.00X10^5$	9
111	18.1.96	8471	$0.80X10^6$	8
112	27.2.96	8556	$0.75X10^6$	9
113	3.4.96	8577	$1.30X10^6$	8
114	30.5.96	8607	$0.80X10^6$	8
115	11.7.96	8605	$0.64X10^6$	9
116	20.8.96	8616	$1.20X10^6$	8
117	1.10.96	8637	$0.75X10^6$	8
118	4.11.96	8701	$1.00X10^6$	9
119	26.12.96	8783	$0.50X10^6$	9
120	29.1.97	8784	$0.26X10^6$	9
121	27.2.97	8709	0.50×10^6	10
122	3.4.97	8898	1.52×10^6	8
123	2.5.97	8892	1.12×10^6	8
124	17.6.97	8875	0.64×10^6	9
125	29.7.97	8928	0.50×10^5	11
126	5.9.97	8937	1.00×10^4	12
127	18.10.97	8926	0.24×10^6	10
128	3.12.97	8792	1.00×10^5	10
129	21.1.98	8931	0.52×10^6	9
130	6.3.98	9143	1.22×10^6	8

Table- 2

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Chloroquine (3 dose regimen)

BN: AU 29291

DATE REC'D: Oct.1993

QUANTITIY: 500 gm

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.= 518

Base= 320

BLOOD SCHIZONTOCIDAL TEST (X 3 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
5.0	8083	Recrudescence on day 16
5.0	8088	Recrudescence on day 14
7.5	8035	Cured
7.5	8074	Recrudescence on day 18
10.0	8078	Cured
10.0	7992	Cured
7.5*	8083	Cured
7.5*	8088	Cured
10.0*	8074	Cured

* Monkeys retreated after recrudescence at the lower dose.

TABLE- 3

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose validation in Sp. passage 90)

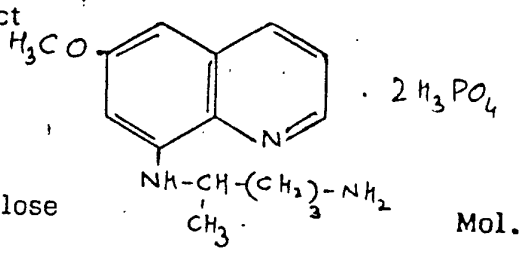
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl Cellulose

ROUTE Oral



Mol.Wt.= 455

Base= 259

PROPHYLACTIC TEST (X 3 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1,78	7772	Cured
1.78	7776	Cured
Control		
-	7775	Patent on day 8
-	7773	Patent on day 9

TABLE- 4

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose revalidation in serial Sp. Passage 86)

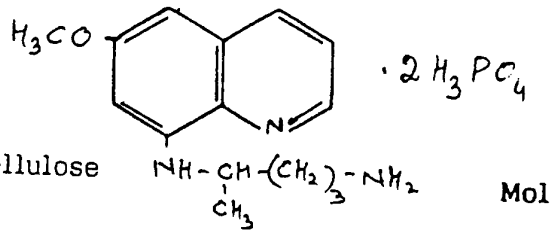
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl cellulose

ROUTE Oral



Mol.Wt.= 455

Base= 259

RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.00	7552	Cured
1.00	7556	Cured
0.316	7548	Relapse on day 29
0.316	7560	Relapse on day 37
Chloroquine Control		
-	7558	Relapse on day 16
-	7578	Relapse on day 19

TABLE- 5

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose revalidation in serial Sp Passage 90)

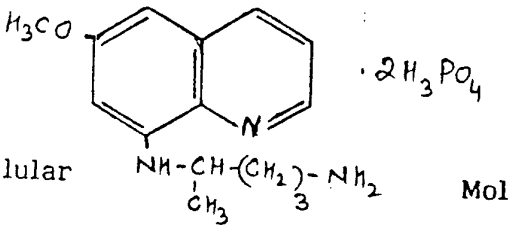
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl Cellular

ROUTE Oral



Mol.Wt.= 455

Base= 259

RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.00	7773	Cured
1.00	7774	Cured
Chloroquine Control	7768	Relapse on day 9

Table- 6

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 142490 (Mefloquine)

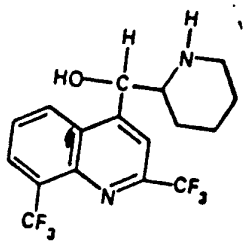
BN: BE. 16387

DATE REC'D: Nov. 1993

QUANTITIY: 1000 mg

VEHICLE: Aqueous

ROUTE Oral



Mol.Wt.= 414.5

Base= 378

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7904	3.16	No parasite clearance
7911	3.16	No parasite clearance
7906	10.0	Cured
7909	10.0	Cured
7903	31.6	Cured
7908	31.6	Cured

Table-7

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Mefloquine

BN: BE 19191

DATE REC'D: July 1994

QUANTITIY: 85 gm

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.= 414.5

Base= 378

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
10.0	8140	• Cured
10.0	8141	Cured

TABLE-8

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 171669 (Halofantrine)

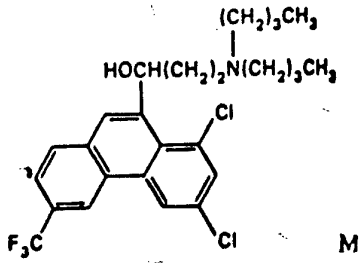
BN: BK 64002

DATE REC'D: Oct. 1988

QUANTITIY: 5 gm

VEHICLE: Aqueous

ROUTE Oral



Mol.Wt.=

Base=

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7912	3.16	Recrudescence on day 12
7921	3.16	Recrudescence on day 14
7919	10.0	Cured
7924	10.0	Cured
7902	31.6	Cured
7905	31.6	Cured

Table-9

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Halofantrine

BN: BK 64002

DATE REC'D: July 1994

QUANTITIY: 50gm

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.=

Base=

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
10.0	8080	Cured
10.0	8081	Cured

Table- 10

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Halofantrine

BN: BK 64002

DATE REC'D: July 1994

QUANTITIY: 50 gm

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

Expt.II I BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
5.62	8139	Recrudescence on day 19
5.62	8180	Cured
5.62	8265	Cured
5.62	8273	Cured
10.0	8138	Cured
10.0	8181	Cured

Table-11

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 242511

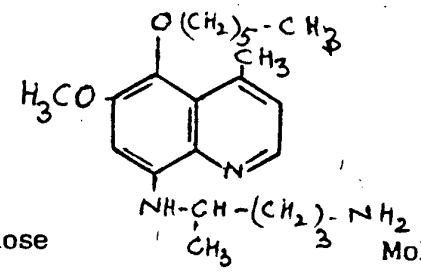
BN: BL 09412

DATE REC'D:

QUANTITIY: 2 gm

VEHICLE: Methyl cellulose

ROUTE Oral



Mol.Wt.= 571

Base= 373

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7907	0.316	Recrudescence on day 16
7910	0.316	Recrudescence on day 16
7913	1.0	cured
7923	1.0	Cured
7918	3.16	Cured
7922	3.16	Cured

Table- 12:

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 242511

BN: BL 09412

DATE REC'D:

QUANTITIY: 2 gm

VEHICLE: Methyl Cellulose Mol.Wt.= 571

ROUTE Oral Base= 373

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.0	8075	Cured
1.0	8079	Cured

TABLE- 13

Comparison of the blood schizontocidal activity of WR 238605 and primaquine against trophozoite induced P.cynomolgi B and P. fragile in rhesus monkeys.

Dose mg(base)/ kgx7 days	Total course dose mg(base)/kg	No. of monkeys treated	Response to treatment	
			No. of monkeys cured	No.of monkeys showing recrudescence(on day)
A. Plasmodium cynomolgi B Infection				
WR238605				
0.316	2.21	4	0	4 (7,13,18,20)
1.00	7.00	12	10	2 (20,26)
3.16	22.12	6	6	0
Primaquine				
1.00	7.00	2	0	2 (10,12)
3.16	22.12	4	0	4 (13,15,16,19)
10.00	70.00	4	1	3 (15,24,28)
B. Plasmodium fragile infection				
WR 238605				
0.316	2.21	4	0	4 (16,19,24,28)
1.00	7.0	11	10	1 (36)
3.16	22.12	4	4	0
Primaquine				
1.00	7.00	2	0	2 (13,16)
3.16	22.12	3	1	2 (17,20)
10.00	70.00	3	2	1 (18)

TABLE- 1r

Gametocytocidal activity of compound WR 238605 in the *P. cynomolgi* - *A. stephensi* - rhesus monkey model

DOSE (Mg/Kg) AT 0 Hr.	TIME OF MOSQUITO FEEDING	PARASITAEMIA/MM^3 ASEXUAL	PARASITAEMIA/MM^3 GAMETO-CYTAEMIA	DAY 7 OOCYST RECORD NO. OF MOSQUITO INFECTED/ DISSECTED (% INFECTI-VITY)	DAY 7 OOCYST RECORD OOCYST NUMBER (MEAN± SD)
1.00	-1Hr.	23112	749	32/37 (86.49%)	33.16±22.18
	+6Hr.	-	-	31/36 (86.11%)	42.26±23.76
	+24Hr.	26215	533	31/39 (79.49)	25.77±17.63
	+48Hr.	7383	321	0/30 (Nil)	-
1.00	-1Hr.	39055	1391	32/40 (80.0)	17.13±10.01
	+6Hr.	-	-	32/44 (72.73%)	13.69±7.20
	+24Hr.	28248	321	0/31 (Nil)	-
	+48Hr.	5992	107	0/23 (Nil)	-
1.00	-1Hr.	48384	6832	25/34 (73.53%)	32.12±13.62
	+6Hr.	-	-	32/40 (80.0%)	31.06±12.73
	+24Hr.	42448	3256	36/48 (75.0%)	30.86±13.81
	+48Hr.	20832	1008	0/25 (Nil)	-
2.00	-1Hr.	54805	2938	43/53 (81.13%)	28.91±18.43
	+6Hr.	-	-	47/58 (81.03%)	27.13±16.30
	+24Hr.	26555	1243	0/38 (Nil)	-
2.00	-1Hr.	42619	2398	25/39 (64.10%)	18.40±10.19
	+6Hr.	-	-	30/48 (62.5%)	17.83±6.80
	+24Hr.	26487	1199	0/36 (Nil)	-
2.00	-1Hr.	42036	3503	22/27 (81.48%)	35.32±13.34
	+6Hr.	-	-	28/34 (82.35%)	26.89±11.19
	+24Hr.	21344	2668	0/31 (Nil)	-
4.00	-1Hr.	73902	3390	28/33 (84.85%)	29.75±12.41
	+6Hr.	-	-	31/36 (86.11%)	38.16±19.28
	+24Hr.	47008	1808	0/30 (Nil)	-

Table-15:

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 (Shorter 3 dose regimen)

BN: BK 73252

DATE REC'D: July 1994

QUANTITIY: 10 gm

VEHICLE: Methyl Cellulose

ROUTE Oral

Mol.Wt.= 531

Base= 463

RADICAL CURATIVE TEST (X 3 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
Expt. I		
0.50	8142	Relapse on day 25
0.50	8144	Relapse on day 43
1.00	8076	Cured
1.00	8146	Cured
2.00	8054	Cured
2.00	8116	Cured
-	8077	Relapse on day 30
Expt. II		
0.75	8424	Cured
0.75	8427	Cured
0.75	8433	Cured
Chloroquine Control	8432	Recrudescence on day 16

Monkeys were concurrently administered chloroquine @ 10.0 mg(base)/kg X 3 days.

Table- 16.

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI - RHESUS MONKEY
SPOROZOITE INDUCED TEST

WR: WR 238605 (without companion blood schizontocide).

BN: BK 73252

DATE REC'D:

QUANTITY:

VEHICLE: Aqueous

ROUTE: Oral

RADICAL CURATIVE TEST (X 7 DAYS)

DOSE (mg/kg)	MONKEY NO.	RESULT
1.00	8682	Cured
1.00	8791	Cured
1.00	8694	Cured
3.16	8785	Cured
3.16	8793	Cured
3.16	8795	Cured
Controls		
0.316 + 5.0 mg/kg chloroquine	8698	Cured
5.0 mg/kg chloroquine	8789	Relapse day 10

Table-17.

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI - RHESUS MONKEY
SPOROZOITE INDUCED TEST

WR: WR 238605 (Without companion blood schizontocide)

BN: BN 65479

DATE REC'D: August 1997

QUANTITY:

VEHICLE: Aqueous

ROUTE: Oral

RADICAL CURATIVE TEST (X 7 DAYS)

DOSE (mg/kg) (x 7)	MONKEY NO.	RESULT
1.0	8924	Cured
1.0	8947	Cured
1.0	8983	Cured

Table- 18

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: **WR 238605 + Halofantrine combination**

BN: BK 73252 , BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.=

Base=

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
WR 238605 + Halofantrine		
0.316 + 1.00	8264	Recrudescence on day 23
0.316 + 1.00	8275	Recrudescence on day 20
0.316 + 3.16	8274	Cured
0.316 + 3.16	8276	Cured
0.316 + 5.62	8271	Cured
0.316 + 5.62	8272	Cured

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Table- 19 : P.cynomolgi- Rhesus Monkey Model

Blood Schizontocidal Activity of Halofantrine and WR 238605 combination (Summarized data)

Treatment Regimen mg/kg x 7 days			No. of* monkeys treated	Response to treatment	
Halofantrine	+	WR 238605		Number** protected	Number Recrudesced (on day)
1.00	+	0.316	2	0	2 (20, 23)
3.16	+	0.316	2	2	-
5.62	+	0.316	2	2	-
10.00	+	-	4	4	-
5.62	+	-	4	3	1 (19)
3.16	+	-	2	-	2 (12, 14)
-	+	3.16	2	2	-

* Treatment administered orally once daily for seven consecutive days.

** Monkeys which did not show any recrudescence upto day 60 post treatment were recorded as protected.

Table- 20:

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 + Halofantrine combination

BN: BK 73252 + BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

Expt.1. RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)			MONKEY NO.	RESULT
WR 238605 + Halofantrine				
0.316	+	3.16	8143	Cured
0.316	+	3.16	8149	Cured
0.316	+	5.62	8145	Cured
0.316	+	5.62	8147	Cured
0.316	+	10.0	8114	Cured
0.316	+	10.0	8115	Cured

Table- 21:

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 + Halofantrine

BN:

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

RADICAL CURATIVE TEST (X 7 day)

Expt.II

DOSE mg/kg(base)	MONKEY NO.	RESULT
WR 238605 + Halofantrine		
0.316 + 1.78	8243	Relapse on day 26
0.316 + 1.78	8244	Cured
0.316 + 3.16	8238	Cured
0.316 + 3.16	8241	Cured
0.316 + 5.62	8237	Cured
0.316 + 5.62	8242	Cured
0.10 + 10.0	8245	Relapse on day 13
0.10 + 10.0	8246	Relapse on day 15
0.316 + -	8239	Relapse on day 26

Table- 22:

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 + Halofantrine Combination

BN: BK 73252 : BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous

ROUTE Oral

Mol.Wt.=

Base=

EXPT.III RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
WR 238605 + HAlofantrine		
0.316 + 3.16	8310	Cured
0.316 + 3.16	8315	Cured
0.10 + 10.00	8313	Cured
0.10 + 10.00	8316	Relapse on day 39
- 10.00	8303	Relapse on day 15
- 10.00	8305	Relapse on day 14

TABLE- 23: : *P. cynomolgi*- Rhesus Model

Anti- Relapse Activity of Halofantrine and WR 233605 combination.

Treatment Regimen mg/kg x 7 days			No. of* monkeys treated	Response to treatment	
Halofantrine	+	WR 238605		Number** protected	Number Relapsed (on day)
10.00	+	-	2	-	2 (14, 15)
-	+	0.316	1	-	1 (39)
10.00	+	0.316	2	2	-
5.62	+	0.316	4	4	-
3.16	+	0.316	6	6	-
1.78	+	0.316	2	1	1 (26)
10.00	+	0.10	4	1	3 (13, 15, 39)

* Treatment administered orally (once daily) for seven consecutive days

** Monkeys that did not show any relapse upto day 90 post-treatment were recorded as protected.

TABLE-24;

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI - RHESUS MONKEY
SPOROZOITE INDUCED TEST

WR: WR 238605 + Desbutyl Halofantrine

BN: BN 65479 + BN 78716

DATE REC'D: August 1997

QUANTITY:

VEHICLE: Aqueous

ROUTE: Oral

RADICAL CURATIVE TEST (X 7 DAYS)

DOSE (mg/kg)	MONKEY NO.	RESULT
WR 238605 + Halofantrine		
0.316 + 3.16	8943	Cured
0.316 + 3.16	8984	Cured
0.316 + 3.16	8940	Cured
Control		
- 10.0	8772	Cured

Table- 25:

COMPOUND	:	Mefloquine + WR 238605 Combination
BN	:	BE 16387/BK 73252
Date Received	:	Nov,93
Quantity	:	
Vehicle	:	Aqueous
Route	:	Oral

BLOOD SCHIZONTOCIDAL TEST X 7 DAY

Dose (mg/kg) WR 238605	Mefloquine	Monkey No.	Result
0.316	+ 3.16	8341	Recrudescence on day 25
0.316	+ 3.16	8344	Recrudescence on day 26
0.316	+ 5.62	8343	Cured
0.316	+ 5.62	8345	Cured
-	5.62	8340	Recrudescence on day 17
-	5.62	8342	Recrudescence on day 12

Table- 26:

COMPOUND : Mefloquine +WR 238605 combination

BN : BE 16387/BK 73252

Date Received :

Quantity :

Vehicle : Aqueous

Route : Oral

RADICAL CURATIVE TEST (x7 DAYS)

Dose(mg/kg)			Monkey No.	Result
WR 238605	+	Mefloquine		
0.316		5.62	8394	Cured
0.316	+	5.62	8406	Cured
0.316	+	10.00	8400	Cured
0.316	+	10.00	8408	Cured
Control				
-		10.00	8392	Relapse on day 11
-		10.00	8413	Relapse on day 12

TABLE-27:

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI - RHESUS MONKEY
SPOROZOITE INDUCED TEST

WR: WR 242511 (Without companion blood schizontocide)

BN: BM 05816

DATE REC'D: August 1997

QUANTITY:

VEHICLE: Aqueous

ROUTE: Oral

RADICAL CURATIVE TEST (X 7 DAYS)

DOSE (mg/kg)	MONKEY NO.	RESULT
1.00	9082	Cured
1.00	9083	Cured
1.00	8934	Cured

Table- 28: Blood schizontocidal activity of antihistaminic drugs against multiresistant P.yoelii nigeriensis (MDRI)

Inoculum : 1 x 10^5 parasitised RBC (i.p.)
Host : Swiss mice (20g+2g)
Treatment schedule : Day 0 to+3 (4 doses, oral)

Treatment (x 4 days)	Parasitaemia %													MST (Days)
	Day 4	Daya5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	Survi-val	
Terfenadine (Trexyl 60) 60mg/kg	9.6±2.5 ±1.44	19.6± ±3.18	67±2.82 ±2.00	92±0.0 ±0.0										7.0
Mebhydrolin (Incidal) 100mg/kg	6.5±7.68 3.84	27.25± 35.26 ±17.63	35.75± ±44.16 ±22.08	2.12± 2.65 ±1.88	14±5.65 +4.0	62.5± 3.53± ±2.50	80±0.0± 0.0							8.88
CDRI 73/602 (Antihistaminic Compound)	3.91±5.12 2.09	22.8± 29.3± 13.13	37.0± 34.2 ± 17.13	58.5± 1909± 13.54										6.83
Cyproheptadine (Ciplactin) 40mg/kg	Toxic	00.0± 00	00.0± 00	00.0± 00	00.0± 00	00.0± 00	0.2±0.0 ±00	1.5±0.0 ±00	5.0±0.0 ±00	46.0±0.0				
20mg/kg	0.0±0.0±	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	.08±0.17 ±.07	1.0±2.23 ±1.0	7.0±15.65 7.01	0.13±0.25 0.12	2.0±4.0 2.0	15.0±30.0 15.0	3/6	<16.0
10mg/kg	0.58± 1.4±0.57	3.75± 8.47± 3.47	12.13± 24.6± 10.08	9.64± 12.30 5.57	3.5± 5.60± 3.23	7.66± 6.65± 3.84	26.6± 27.2± 15.72	38.3± 30.9± 17.87						9.83
Control	11.5± 6.24± 3.12	57.5± 28.7± 14.36	63± 25.9± 14.9	83.5± 16.26± 11.53										7.25

Table - 29:

Causal prophylactic activity of antihistaminic agents against sporozoite induced infection of Plasmodium yoelii nigeriensis (N-67) in Swiss mice.

Drug/Dose (mg/kg)	No. of mice	Response to treatment		
		Day of Patency		Percent protection
		Range	Mean ± SE	
Ketotifen				
1.25	10	6-11	8.20±0.51	0
2.5	10	7-10	8.60±0.54	50
5.0	10	-	-	100
Cyproheptadine				
1.25	10	5-6	5.50±0.16	0
2.5	10	5-7	6.13±0.21	20
5.0	10	-	-	100
Azatadine				
10.0	10	7-11	9.00±0.37	0
50.0	10	8-11	10.00±0.32	0
Loratadine				
10.0	10	6-10	8.00±0.35	0
50.0	10	7-11	9.20±0.37	0
Terfenadine				
12.5	10	5-7	6.10±0.22	0
25.0	10	6-10	7.00±0.40	0
50.0	10	-	-	100
Primaquine				
32.0	10	-	-	100
Pyrimethamine				
0.1	10	-	-	100
Control	10	3-5	3.80±0.19	0

- = Mice remained negative during the entire observation period.

Table-30:

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI - RHESUS MONKEY

SPOROZOITE INDUCED TEST

WR: Cyproheptadine

BN: Commercial Sample

DATE REC'D:

QUANTITY:

VEHICLE: Solubilized with Tween 80

ROUTE: Oral

RADICAL CURATIVE TEST X 7 DAYS

DOSE (mg/kg)	MONKEY NO.	RESULT
5.0	8883	Relapse on day 29
5.0	8885	Relapse on day 55

Chloroquine 5.0 mg/kg (base) x 7 days administered as companion blood schizontocidal drug.

Table-31: Blood schizontocidal activity of azithromycin and erythromycin against

Plasmodium yoelii nigeriensis (N-67) in Swiss mice

Drug Dose (mg/kg) x 4 days	No. of mice	Mean percent parasitaemia (±SE) on day					Mice Cured/ Mice treated	Mice surviving on day 28	*Mean survival time±SE (days)
		4	7	14	21	28			
Azithromycin									
15	12	2.08± 0.23	5.58± 0.36	18.33 ± 1.68	5.80± 1.28	Nil	0/12	5	18.86± 1.05
45	12	Nil	Nil	2.83± 0.73	Nil	Nil	3/12	12	
70	12	Nil	Nil	Nil	Nil	Nil	12/12	12	
135	12	Nil	Nil	Nil	Nil	Nil	12/12	12	
Erythromycin									
45	12	2.50± 0.22	8.92± 0.79	42.12± 3.94	Nil	Nil	0/12	2	15.00± 1.35
135	12	1.58± 0.14	6.92± 0.69	35.70± 3.61	Nil	Nil	0/12	4	15.88± 1.11
405	12	1.17 ± 0.11	3.92± 0.38	29.83± 3.29	Nil	Nil	0/12	6	18.33± 0.61
Control	12	11.75 ± 1.20	33.09± 2.86				0/12	0	10.00± 0.57

*MST determined for those animals which died within day 28.

Table-32: . Blood schizontocidal activity of azithromycin against established infection of

Plasmodium yoelii nigeriensis (N-67) in Swiss mice

Dose (mg/kg)	No of mice	Treatment on days	Mean percent parasitaemia (± SE) on day							Mice cured/ Mice treated	Mice surviving on day 28	*Mean survival time± SE (days)
			2	6	8	12	16	21	28			
45	12	2–5	3.25 ± 0.27	4.40 ± 0.80	0.89 ± 0.36	Nil	1.00 ± 0.44	Nil	Nil	5/12	9	6.00 ± 0.47
70	12	2–5	3.17 ± 0.33	2.67 ± 0.75	Nil	Nil	Nil	Nil	Nil	12/12	12	
Control	12	–	3.83 ± 0.26	45.00 ± 5.27	49.00 ± 0.71					0/12	0	6.58 ± 0.39

* MST determined for those animals which died within day 28.

Table-33: Efficacy of azithromycin after 4 day and 7 day treatment regimens against

Plasmodium yoelii nigeriensis (N-67) in Swiss mice

Dose (mg/kg)	No. of mice	*Treatment regimen	Mean percent parasitaemia (±SE) on day					Mice Cured/ Mice treated	Mice surviving on day 28	**Mean survival time±SE (days)
			4	7	16	21	28			
10	12	4d	6.17± 0.55	20.00± 1.77	7.25± 0.96	Nil	Nil	0/12	8	6.00± 0.71
10	12	7d	4.83± 0.55	1.33± 0.45	7.33± 1.24	Nil	Nil	0/12	10	19.00± 0.00
20	12	4d	1.33± 0.19	3.00± 0.53	3.80± 0.44	Nil	Nil	0/12	10	15.00± 0.00
20	12	7d	1.33± 0.19	Nil	0.83± 0.36	Nil	Nil	0/12	12	
40	12	4d	Nil	Nil	4.17± 0.84	Nil	Nil	2/12	12	
40	12	7d	Nil	Nil	Nil	Nil	Nil	12/12	12	
Control	12	--	14.50± 2.37	49.00± 0.71				0/12	0	6.58± 0.39

*4d -Treatment day 0–3; 7d – Treatment day 0–6

**MST determined for those animals which died within day 28

Table-34: ED_{50}/ED_{90} values of antibiotics against Plasmodium yoelii nigeriensis (N-67) in Swiss mice

Drug	ED_{50} (mg/kg)			ED_{90} (mg/kg)		
	Mean	95% Confidence limit		Mean	95% Confidence limit	
		Lower	Upper		Lower	Upper
Azithromycin	**5.00**	4.19	5.97	**28.22**	18.46	43.16
Erythromycin	**38.45**	28.84	51.27	**865.23**	450.91	1660.25
Doxycycline	**9.92**	8.27	11.89	**39.30**	30.28	51.02

Table-35: Causal Prophylactic activity of macrolide antibiotics against sporozoite induced infection of Plasmodium yoelii nigeriensis (N-67) in Swiss mice

Drug/Dose (mg/kg)	No. of mice	Day of patency Mean±SE	No. protected No. treated	Percent Protection
Azithromycin				
25	10	5.00±0.24	0/10	0
50	10	Nil	10/10	100
100	10	Nil	10/10	100
Erythromycin				
135	10	4.50±0.16	0/10	0
405	10	5.90±0.22	0/10	0
Primaquine				
32	10	Nil	10/10	100
Pyrimethamine				
0.1	10	Nil	10/10	100
Control	10	4.30±0.15	0/10	0

Table-36: . Causal prophylactic activity of azithromycin against P. cynomolgi B sporozoite challenge in rhesus monkeys

Drug/Dose (mg/kg)	No. of monkeys	Response to treatment	
		Monkeys protected	Monkeys developed patent infection (on day)
Azithromycin			
25.0	3	0	3 (33, 39, 47)
12.5	2	0	2 (23, 29)
6.25	1	0	1 (21)
Primaquine			
1.0	2	2	
Pyrimethamine			
10.0	2	0	2 (33, 39)
Vehicle control	2	0	2 (10,11)

Table-37: Response of azithromycin against established sporozoite induced infection of *P. cynomolgi* B in rhesus monkeys

Drug Dose (mg/kg)	No. of monkeys treated	No. of monkeys protected	Monkeys relapsed (on day)
Azithromycin			
25.0	2	0	2 (19, 20)
Chloroquine control			
5	1	0	1 (17)
Primaquine control			
1.0	1	1	

Table- 38: Sequential maintainence of P.knowlesi for selection of chloroquine resistant strain by interrupted subcurative therapy.

No.	Monkey No.	Date of inoculation	Exposure to chloroquine			Isolate cryopreserved
			No. of doses	Duration (Days)	Total dose (mg/kg)	
Rh-1	7943	19.1.94	5 doses (0.5-3.0 mg/kg)	8 days	7mg/kg	
Rh-2	7945	28.1.94	25 doses (.2-3 mg/kg)	82 days	23.7 mg/kg	R1- 2.4.94 R2- 26.4.94
Rh-3	8027	20.4.94	32 doses (.5-.2 mg/kg)	75 days	24.5 mg/kg	
Rh-4	8085	4.7.94	11 doses (.5-2 mg/kg)	44 days	10 mg/kg	
Rh-5	8087	17.8.94	4 doses (.5-2 mg/kg)	27 days	5.5 mg/kg	R3- 13.9.94
Rh-6	8282*	22.12.94	13 doses (1-2 mg/kg)	54 days (till 14.2.95)	16 mg/kg	

* Monkey inoculated with cryopreserved sample (R3) of 13.9.94.

able- :

ffects f different concentrations of Verapamil with chloroquine on reversal of drug resistance in P.yoelii nigeriensis train

train: P.yoelii nigeriensis (multi drug resistant), Inoculum : $1x10^6$ parasites; Route: Oral;.Treatment: 4 days (i.e. from day 0 - Day +3)

ug	Dose (day)-+3)	Day 4 Mean±SD±SE	% Parasitaemia Record (Mean±SD) (No. of mice surviving)						
			Day 7	Day 14	Day 18	Day 21	Day 24	Day 28	MST
ntrol	-	9.38±4.7±1.9 (6)							6.0
...roquine	8mg/kg	Nil (8)	3.32±4.5 ±1.6 (8)	7.5±0.0	7.0±0.0				10.75
rapamil	25mg/kg	18.7±11.6 (7)	32.9±15.9 (4)						8.4
rapamil Chloroquine	25mg/kg + + 8mg/kg	Nil (8)	0.3±0.5 ±0.2 (8)	8.6±10.5 ±5.3 (4)	3.3±4.7 ±3.3 (2)	0.2±0.3 ±0.2 (2)	Nil (1)	Nil (1)	12.25
rapamil Chloroquine	10mg/kg + + 8mg/kg	Nil (8)	3.7±9.8± ±3.5 (8)	5.2±2.9 ±2.1 (2)	8.0± (1)	0.4± (1)	-	-	12.63
...pamil ...roquine	1.0mg/kg + 8mg/kg	Nil (8)	5.3±7.9 ±2.8 (9)						9.8
...pamil ...roquine	0.5mg/kg + 8mg/kg	Nil (7)	1.4±3.5 ±2.5 (7)						9.13

Table- 40:

Effects of different concentration of Verapamil with chloroquine on reversal of drug resistant
Strain: P.yoelii nigeriensis; Inoculum: $7x10^6$; Routeof drugs: Oral, Dose time; 4 days (day 3-6)

Drug	Dosemg/kg (day 3-6)	Day 4	& Parasitaemia (No. of mice surviving) Mean±AS) Day 7	Day 10	Day 14	Day 18	Day 21	Day 24	Day	MST
Control Av. 2 5% of 25 mice		Nil (5)	2.68±0.8 ±0.6 (2)							7.4
Chloroquine	8	Nil (7)	0.3±0.8 ±0.3 (7)	6.3±3.8 ±1.5 (6)	4.65±4.5 ±2.2(4)	1.8±2.8 ±1.6 (4)	0.2±0.2 ±0.1 (4)	Nil (4)	Nil (3)	21.14
Chloroquine	16	Nil (7)	0.4±0.8 ±0.3 (7)	2.9±3.5 ±1.3 (7)	1.37±0.3 ±0.2 (3)	0.2±0.3 ±0.2 (3)	0.1±0.2 ±0.1 (3)	Nil (3)	Nil (3)	19.14
Verapamil	25	- (7)	17.5±8.9 (3)	20.0±0.0 (1)						8.3
Verapamil + Chloroquine	5	Nil (7)	0.14±0.4 ±0.14 (7)	8.5±6.8 ±2.6 (7)	2.8±4.4 ±1.8 (6)	1.23±2.8 ±1.2 (6)	Nil (6)	Nil (6)	(6)	25.75
Verapamil + Chloroquine	8	Nil (30)	Nil (3)	6.4±4.9 ±2.8 (3)	21.7±22.5 ±12.9 (3)	2.8±3.9 ±2.8 (2)	Nil (2)	Nil (2)	Nil (2)	24.60
Verapamil + Chloroquine	8	Nil (6)	Nil (6)	2.2±1.6 ±0.65(6)	4.8±7.3 ±3.3(5)	0.7±1.4 ±0.7(4)	Nil (4)	Nil (4)	Nil (4)	23.67
Verapamil + Chloroquine	8	Nil (6)	0.01±0.02 ±0.01 (7)	4.5±2.8 ±1.13 (6)	13.2±12.0 ±6.9 (3)	1.3±1.8 ±1.3 (2)	Nil (1)	Nil (1)	Nil (1)	15.71

Tabel- 41

Curative efficacy/chloroquine resistant reversal activity of nifedepin with chloroquine.

Drug	Dose	Drug schedule	Parasitaemia% Mean±SE (no. of mice surviving) Day 4	Day 7	Day 14	Day 18	Day 21	Day 28	MST
Nifidepine + Chloroquine	25mg/kg + 8mg/kg	3-7 days	(7)	0.5±0.5 (7)	7.35±1.79 (7)	1.75±1.03 (4)	Nil (3)	Nil (3)	20.9
Nifidepine + Chloroquine	15mg/kg + 8mg/kg	3-7	(7)	0.07 ±0.07	6.27 ±2.61	0.083 ±0.06	Nil (3)	Nil (3)	24.7
Nifidepine + Chloroquine	10mg/kg + 8mg/kg	3-7	(6)	Nil (6)	1.16±1.16 (6)	1.27±1.27 (6)	Nil (3)	Nil (3)	24.8
Nifidepine + Chloroquine	5mg/kg +	3-7	(7)	Nil (7)	2.20±2.20 (3)	5.6±0.0 (1)	-	-	14.4
Nifedepine	25mg/kg	3-7	(7)	36.25 ±13.29 (2)	-	-	-	-	7.1
Chloroquine	8mg/kg	3-7	(7)	0.29 ±0.28 (7)	4.67 ±2.23 (4)	1.8 ±1.60 (4)	0.15 ±0.09 (4)	Nil	21.14
Control	- -	-	(5)	26.8±0.64 (2)					7.4

Table- 42:

Evaluation of WR 238605 with chloroquine against *P.yoelii nigeriensis* (Multi Drug Resistant) for resistance Reversal study

Treatment schedule D o→D +3, Route of drug administration (Oral) Av. wt. of mice = 20 gm

Drug	Dose mg/kg	No. of mice	Mean of % parasitaemia on days							M.S.T.
			Day 4	Day 7	Day 10	Day 14	Day 21	Day 28	No. of mice survived	
Control	-	5	25.81 ± 3.2							6.2±.82
Chloroquine + WR 238605	8+0.5	5	0.75 ± .35	1.16 ± .52	1.45 ± .52	2.7 ± .98	-ve	-ve	2	19.4±9.0
Chloroquine + WR 238605	4+0.5	5	0.88 ± .10	.98 ± .20	1.8 ± .75	2.5 ± .70	2.5 ±0			14.4±5.9
Compound WR 238605	0.5	5	11.33 ±3.2							5.0± .83
Chloroquine	8.0	5	1.48 ±.48	2.0 ±.57	2.25 ±.25	2.55 ±.70	-ve	-ve	2	17.8±9.4
Chloroquine	4.0	5	2.44 ±.72	2.4 ±.73	4.33 ±.28	6.5 ±0				12.8±4.2

Table- 45:

Evaluation of mefloquine with WR 238605 for study of drug reversal activity against P.yoelii nigeriensis(MDR)

Treatment schedule- D_0-D+3 Av.wt. of mice= 20 gm

Drug	Dose mg/kg	No.of mice	Mean of % parasitaemia on days				MST
			Day 4	Day 7	Day 10	Day 14	
Mefloquine	1.0	5	3.8	7.0	-	-	6.6
Mefloquine	2.0	5	3.7	4.5	4.5	-	9.2
Mefloquine	4.0	5	0.24	.58	.58	1.3	14.0
Mefloquine	8.0	5	0.24	3.45	.83	1.29	15.0
WR 238605	0.5	5	26.6	-	-	-	6.2
WR 238605+Mefloquine	0.5+1.0	5	1.8	5.0	-	-	6.6
WR 238605+Mefloquine	0.5+2.0	5	1.7	2.25	4.5	5.0	10.0
WR 238605+Mefloquine	0.5+4.0	5	1.06	1.25	1.58	2.2	11.0
WR 238605+Mefloquine	0.5+8.0	5	0.22	.32	.87	1.15	13.2
Control	-	5	27.6	-	-	-	5.8

Table- 44:

Evaluation of WR 238605 with mefloquine for drug reversal study against P.yoelii nigeriensis (multi drug resistant strain)

Treatment therapeutic (D+3 to D+6)

Drug	Dose mg/kg	No.of mice	% Parasitaemia on days						No.of mice survival on days(50)	MST (Days)
			3	4	7	10	14	35		
Control			6.7±2.3	16.66±3.2	-	-	-	-	-	5.0
WR 238605 + Mefloquine	0.5±16.0	6	6.0±1.5	1.5±	0.71±.20	1.05±.44	1.2±.44	.15±.06	2	29.33
WR 238605 + Mefloquine	0.5±8.0	6	4.7±.51	1.2±.59	0.65±.36	1.5±.58	.71±.64	0.36±.15	3	35.33
WR 238605 + Mefloquine	0.5±4.0	6	4.23±1.06	1.13±.32	0.73±.29	1.21±.27	2.1±.22	0	1	21.5
WR 238605	0.5	6	5.66±1.5	14.95±3.3	-	-	-	-	-	5.16
Mefloquine	16.0	6	5.72±1.5	1.25±.22	0.68±.82	1.75±.82	1.66±.42	0.22±.17	4	37.66
Mefloquine	8.8	6	4.66±1.3	1.63±.62	1.23±.28	2.08±.90	3.7±.90	0.36±.15	3	31.50
Mefloquine	4.0	6	4.53±1.7	1.68±.64	1.15±.31	1.33±.25	1.45±.41	0.30±0	1	26.0

Table- 45:
Evaluation of Quinidine with chloroquine against multi drug resistant P.yoelii nigerinsis

Treatment Therapeutic D+3 to D+6

Drug	Dose mg/kg	No.of mice	% Parasitaemia on days							MST (Days)
			3	4	7	10	14	30	No.of mice survival	
Control	-	6	2.58±.58	15.16±.98	-	-	-	-		7.16
Quinidine + Chloroquine	25+8	6	2.5±.40	3.25±.68	0.78±.36	0.33±.08	.76±.40	0	4	24.16±9.08
Quinidine + Chloroquine	25+4	6	2.58±.37	3.66±.40	.75±.20	0.95±.08	1.76±.79	0	4	27.0
Quinidine + Chloroquine	15+8	6	2.57±.49	4.08±1.1	.48±.11	0.91±.17	1.6±.5	0	4	23.833
Quinidine + Chloroquine	15+4	6	2.58±.36	4.53±.49	.71±.06	.75±.31	1.6±.05	0	4	25.33
Quinidine + Chloroquine	10+8	6	2.50±.40	4.58±.49	1.3±.36	.83±.20	0.92±.50	0	4	23.83
Quinidine + Chloroquine	10+4	6	2.33±.40	5.5±1.1	2.0±.15	5.2±.35		-	-	12.83
Quinidine	25.0	6	2.08±.25	5.25±.82	5.75±.15	5.95±.15	-	-	-	11.83
Quinidine	15.0	6	2.5±.40	4.2±1.3	1.0±.26	1.5±.05	1.25±.50	-	-	13.833
Quinidine	10.0	6	2.5±.40	5.9±1.6	4.95±.15	4.55±.50	-	-	-	10.0
Chloroquine	8.0	6	2.16±.25	5.5±.15	.88±.34	5.2±1.5	-	-	-	11.55
Chloroquine	4.0	6	2.33±.25	7.33±1.2	5.5±1.5	6.0±0	7.5±0	-	-	13.833

Table- 46 Establishment of drug resistant isolates of Plasmoidum yoelii nigeriensis (N-67) in Swiss mice model.

Isolate resistant to	Curative dose in parent strain (mg/kg x 4 days)	Resistance level (mg/kg x 4 day)	Stability after transmission through mosquitoes
Chloroquine	16 mg/kg	>128 mg/kg	Stable
Mefloquine	8 mg/kg	>128 mg/kg	Stable
Halofantrine	4 mg/kg	>128 mg/kg	Stable
Pyrimethamine	4 mg/kg	> 48 mg/kg	Stable

Table- 47 Resistance modulation studies against CHLOROQUINE RESISTANT isolates of P.yoelii nigeriensis (N-67)- Swiss mice model.

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ± SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	16	28		
Vehicle control	12	9.17 ± 1.83	23.60± 4.62			Nil	8.67±2.06
CHL- 16.0	12	2.00± 0.63	3.83± 0.98	40.00± 12.75	Nil	5	17.00±1.42
CHL - 16.0 + Cyproheptadine- 10.0	12	Nil	Nil	Nil	Nil	12	
CHL - 16.0 + Amitryptiline - 50.0	12	Nil	1.02± 0.60	14.00± 10.71	Nil	Nil	18.50±0.70
CHL- 16.0 + Verapamil - 50.0	12	0.07± 0.05	3.33± 1.03	19.67± 6.77	Nil	8	25.50±0.70
CHL - 16.0 + Amantidine 50.0	12	2.50± 0.55	5.67± 1.03	40.00± 14.58	Nil	8	18.00±4.24

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Table - 48 Resistance modulation studies against MEFLOQUINE RESISTANT isolate of P.yoelii nigeriensis (N-67)- Swiss mice model

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ±SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	16	28		
Vechile control	12	7.50±1.52	27.50±7.15			Nil	11.00±2.37
Mefloquine 8.0	12	2.17±0.75	8.33±2.58	32.00±13.95	Nil	4	16.50±2.08
MFQ - 8.0 + Cyproheptadine 10.0	12	Nil	Nil	0.50	Nil	12	
MFQ - 8.0 + Amitryptiline - 50.0	12	Nil	2.17±0.75	30.00±8.63	Nil	8	17.50±2.12
MFQ - 8.0 + Verapamil - 50.0	12	0.02±0.04	5.17±1.47	39.60±7.40	Nil	10	16.0±0.00
MFQ - 8.0 + Amantidine - 50.0	12	3.00±0.89	6.83±1.17	40.00±7.90	Nil	8	18.50±3.54

Animals inoculated with 1×10^7 parasites on Day 0

Observations upto day 28 post inoculation.

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Table-49 Resistance modulation studies against HALOFANTRINE RESISTANT isolate of P.yoelii nigeriensis (N-67)

Swiss mice model

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ±SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	16	28		
Vehicle control	12	5.33±1.75	15.33±2.80	-		Nil	12.17±3.19
Halofantrine - 4.0	12	3.00±1.26	10.83±4.12	39.25±9.78	Nil	4	16.00±3.92
Halofantrine - 4.0 + Cyproheptadine - 10.0	12	Nil	Nil	Nil	Nil	12	
Halofantrine - 4.0 + Amitryptiline - 50.0	12	0.69±0.49	1.33±0.52	16.17±15.03	Nil	2	19.50±2.12
Halofantrine - 4.0 + Verapamil - 50.0	12	0.05±0.05	3.50±1.05	19.83±3.49	Nil	8	25.00±0.00
Halofantrine - 4.00 + Amantidine - 50.00	12	2.83±0.98	6.83±2.14	42.20±15.30	Nil	10	16.00±0.00

Animals inoculated with 1×10^7 parasites on day 0

Observations upto day 28 post inoculation

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Table-50 : Resistance modulation studies against PYRIMETHAMINE RESISTANT isolate of *P.yoelii nigeriensis* (N-67) Swiss mice model

Treatment Regimen mg/kg (Day 0-3)	Number treated	Mean % Parasitaemia ±SD on day				Mice surviving on day 28	Mean survival time ±SD
		4	7	10	28		
Vehicle control	12	7.83±1.47	20.00±7.90	36.00±19.79		Nil	9.17±1.60
Pyrimethamine - 4.0	12	6.00±1.26	13.00±2.68	32.50±10.40		Nil	12.33±3.08
Pyrimethamine - 4.0 + Cyproheptadine - 10.0	12	2.83±0.98	11.67±5.32	35.00±11.18		Nil	12.00±2.00
Pyrimethamine - 4.0 + Amitryptline - 50.0	12	3.33±0.82	10.33±4.51	35.00±7.07		Nil	8.33±3.50

Table-51 : P.Knowlesi-Rhesus Monkey Model

Modulation of mefloquine resistance by cyproheptadine

Treatment Regimen			Number of monkeys treated (Initial Parasitaemia	Response to treatment	
Mefloquine mg/kg x 3 days)	+	Cyproheptadine mg/kg x 5 days days)		Number* protected	Number Recrudesced (on day)
40.0	+	-	2 (03, 1.8%)	-	2(11, 16)
20.0	+	-	2 (0.3, 2.7%)	-	2 (9, 9)
20.0	+	10.0	2 (2.2, 2.8)	2	
20.0	+	5.0	3 (1.4, 1.6, 2.2%)	3	-
20.0	+	2.5	4 (1.3, 1.4,1.7,3.7%)	2	2 (13, 14)
20.0	+	1.25	6 (0.4,0.4,1.6,1.9 2.2,2.5%)	2	4 (9,11,11,16)
20.0	+	0.62	2 (2.3, 2.5%)	1	1 (9)
10.0	+	5.0	2 (0.8, 1.2)	2	-
10.0	+	2.5	2 (1.8, 2.6)	2	2 (4, 8)

* Monkeys which did not show recrudescence upto day 60 post treatment were recorded as 'Protected'

Table-52 : *In vitro* cultivation of *P. knowlesi*: Comparison of growth in different cultures vessels

Culture Vessel	Volume of media (ml)	Height of media (cm)	Haema-tocrit (%)	Mean percent parasitaemia ± S.E.				Multiplication rate at		
				0 h	24 h	48 h	72 h	24 h	48 h	72 h
96 well plate	0.1	0.3	6	0.5	0.8± 0.03	1.1± 0.05	2.5± 0.11	1.6	2.2	5.0
24 well plate	0.57	0.3	6	0.5	1.5± 0.03	2.0± 0.12	3.4± 0.09	3.0	4.0	6.8
35 mm petri-dish	2.6	0.3	6	0.5	0.9 ± 0.09	1.8± 0.1	3.5± 0.15	1.8	3.6	7.0
25 cm^2 tissue culture flask	7.2	0.3	6	0.5	0.9± 0.03	1.7± 0.05	3.8± 0.11	1.8	3.4	7.6

Table-53: : *In vitro* cultivation of *P. knowlesi*: Comparison of growth* at different haematocrits

Haemato-crit (%)	Mean percent parasitaemia ± S.E.				Multiplication rate at		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h
3	0.6	1.2±0.06	2.3±0.14	2.7±0.06	2.0	3.8	4.5
6	0.6	1.4±0.08	2.7±0.29	4.9±0.15	2.3	4.5	8.2
12	0.6	1.5±0.22	4.0±0.09	5.7±0.31	2.5	6.7	9.5
18	0.6	1.2±0.11	4.1±0.23	4.5±0.37	2.0	6.8	7.5

*Cultured in 24 well plates in triplicate well

Table-54 : *In vitro* cultivation of *P. knowlesi*: Comparison of growth* with respect to frequency of media renewal

Interval between medium renewal	Per cent parasitaemia ± S.E.					Multiplication rate at			
	0 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
24 h	1.2	1.9± 0.03	3.6± 0.21	5.3± 0.08	5.3± 0.24	1.6	3.0	4.4	4.4
12 h	1.2	2.1± 0.62	4.3± 0.62	5.1± 0.33	5.9± 0.31	1.8	3.6	4.3	4.9

*Cultured in 24 well plates in triplicate wells.

Table-55 : *In vitro* cultivation of *P. knowlesi*: Comparison of growth* in variable percentage of rhesus serum

Percent of serum in RPMI medium	Per cent parasitaemia ± S.E.					Multiplication rate at			
	0 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
0	0.8	0.6± 0.09	0.3± 0.03	0	–	0.8	0.4	0	–
10	0.8	1.5± 0.14	4.7± 0.28	5.5 ± 0.05	5.2± 0.27	1.9	5.9	6.9	6.5
20	0.8	2.2± 0.11	6.6± 0.33	11.8± 0.46	11.5± 0.86	2.8	8.3	14.8	14.4
50	0.8	2.6± 0.09	8.9± 0.60	13.3± 0.42	10.9± 0.63	3.3	11.1	16.6	13.6
100	0.8	2.6± 0.16	5.4± 0.42	4.7± 0.25	4.5± 0.21	3.3	6.8	5.9	5.6

*Cultured in 24 well plates in triplicate wells

Table-56: ***In vitro* cultivation of *P. knowlesi*: Comparison of growth* in erythrocytes after storage for variable intervals**

Duration of storage at 4°C (Days)	Per cent parasitaemia ± S.E. at				Multiplication rate at		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h
Fresh	1.2	2.4± 0.05	7.2± 0.41	9.7± 0.15	2.0	6.0	8.1
2	1.2	2.8± 0.12	6.5± 0.04	10.0± 0.19	2.3	5.4	8.3
4	0.9	2.4± 0.09	5.0 ± 0.29	7.0± 0.36	2.7	5.6	7.8
15	1.2	1.6± 0.10	2.9± 0.17	3.3± 0.26	1.3	2.4	2.8
30	1.1	1.3± 0.05	1.4± 0.15	1.0± 0.59	1.2	1.3	0.9
40	1.2	1.0± 0.07	0.7± 0.01	0.6± 0.08	0.8	0.6	0.5

* Cultured in 24 well plates in triplicate wells.

Table-57: ***In vitro* cultivation of *P. knowlesi*: Comparison of growth* in rhesus erythrocytes stored at 4°C versus 37°C**

Storage of erythrocytes		Per cent parasitaemia ± SE at				Multiplication rate at		
Duration (Days)	Tempera-ture	0 h	24 h	48 h	72 h	24 h	48 h	72 h
2	Fresh	1.2	2.4±0.05	7.2±0.41	9.7±0.15	2.0	6.0	8.1
	4°C	1.2	2.8±0.12	6.5±0.44	10.0±0.19	2.3	5.4	8.3
	37°C	1.2	1.8±0.24	3.2±0.26	5.8±0.43	1.5	2.7	4.8
4	4°C	0.9	2.4±0.09	5.0 ±0.28	7.0±0.36	2.7	5.6	7.8
	37°C	0.9	1.6±0.17	2.4±0.18	1.9±0.09	1.8	2.7	2.1
15	4°C	1.2	1.6±0.10	2.9±0.16	3.3±0.26	1.3	2.4	2.8
	37°C	1.2	2.15±0.17	2.9±0.27	0.92±0.16	2.1	2.4	0.8

*Cultured in 24 well plates in triplicate wells.

Table-58: **: Continuous cultivation* of *P. knowlesi* using candle jar method**

Day	Percent Parasitaemia				Number of times diluted	Cumula-tive dilution	Multiplication rate		Cumulative increase (fold)
	Ring	Trophozoite	Schizont	Total			Interval between dilutions (Days)	Increase (fold)	
0	1.4	-	-	1.4					
1	1.5	0.1	-	1.6					
2	1.8	-	-	1.8	1.5	1.5	2	1.3	1.3
3	2.4	0.2	0.4	3.0					
4	1.2	1.0	0.2	2.4					
5	1.6	0.3	0.3	2.2	1.5	2.3	3	1.8	2.3
6	3.6	-	-	3.6					
7	3.1	0.1	0.1	3.3	2	4.5	2	2.3	5.4
8	1.9	0.1	0.2	2.2					
9	3.9	-	0.2	4.1	1.5	6.8	2	2.5	13.5
10	4.1	0.2	0.4	4.7					
11	3.3	1.2	0.5	5.0	1.5	10.1	2	1.8	24.2
12	2.7	0.4	0.3	3.4	2	20.3	1	1.1	26.6
13	2.8	0.5	0.2	3.5	4	81.0	1	2.1	55.9
14	1.1	0.7	0.6	2.4					
15	2.1	0.7	0.6	3.4	3	243	2	3.9	218.2
16	1.2	0.5	0.3	2.0					
17	3.0	0.4	0.1	3.5					
18	6.1	0.7	1.2	8.0					
19	4.4	0.1	0.2	4.7	2	486	4	4.1	894.6
20	1.1	1.1	0.2	2.4					
21	1.7	1.6	0.6	3.9	3	1458	2	1.7	1520.8
22	2.4	1.4	0.4	4.2					
23	4.0	0.7	0.8	5.5	2	2916	2	4.2	6387.3
24	5.1	0.2	0.6	5.9	1.5	4374	1	2.1	13413.3
25	5.7	1.6	1.0	8.3					
26	5.5	0.1	0.4	6.0	2	8748	2	1.5	20119.9
27	4.0	0.9	0.8	5.7					
28	5.8	0.7	0.3	6.8	3	26244	2	2.3	46275.7
29	2.3	0.5	0.5	3.3					
30	4.2	1.1	0.3	5.6					
31	3.9	0.6	0.3	4.8					
32	5.3	1.0	0.7	7.0					
33	3.4	1.4	0.7	5.5					
34	3.8	1.3	0.4	5.5	6	157464	6	2.4	111061.7
35	0.6	0.1	0.3	1.0					

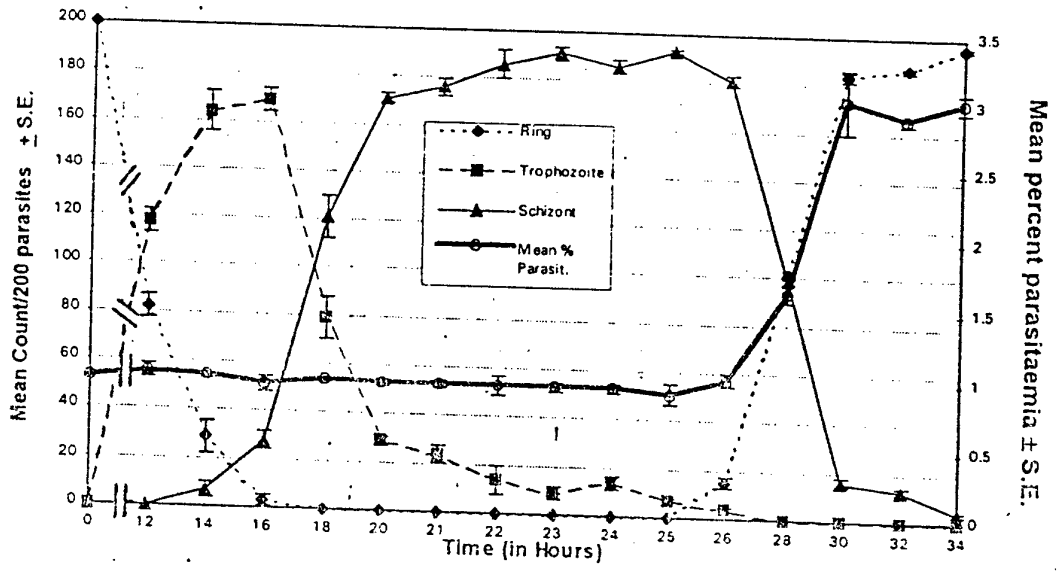
*Culture vessel: 2.5 cm flat bottomed, loose capped glass vessel.

Average multiplication rate per cycle = 1.03

Table-59: . Optimization of *in vitro* drug sensitivity assay against *P. knowlesi*: Differential count of intraerythrocytic stages during 34 hour duration

Hours	Mean count/200 parasites ± S.E.						Mean percent parasitaemia ± SE
	Ring		Trophozoite		Schizont		
	Early	Late	Early	Late	2 nucleated	multi nucleated	
							
0	200	0	0	0	0	0	0.93
12	0	82±4.8	89±9.7	29±7.3	0	0	0.97±0.05
14	0	29±6.5	84±2.5	80±7.1	7±3.3	0	0.95±0.02
16	0	3±2.4	47 ± 5.0	122±6.9	25±1.9	3±1.36	0.9±0.05
18	0	0	4 ± 0.27	75±8.8	86±7.4	35±2.1	0.94±0.02
20	0	0	0	29±2.2	49±6.1	122±3.9	0.93±0.03
21	0	0	0	24±3.6	32±0.94	144±4.0	0.93±0.03
22	0	0	0	14±5.7	25±3.8	161±6.6	0.93±0.07
23	0	0	0	9±2.9	15±0.98	176±3.5	0.92±0.04
24	0	0	0	14±2.6	4±0.47	182±2.5	0.92±0.04
25	0	0	0	7±0.72	4±0.72	189±0.47	0.89±0.08
26	15±2.4	0	0	4±0.54	0	181±2.5	1.0±0.05
28	101±1.7	0	0	0	0	99±1.7	1.63±0.07
30	184±1.9	0	0	0	0	16±1.9	3.03±0.24
32	187±1.25	0	0	0	0	13±1.2	2.9±0.03
34	196±0.54	0	0	0	0	4±0.54	3.03±0.07

*Cultured in 96 well plates in triplicate wells.



: Differential count of *P. knowlesi* intra erythrocytic stages during 34 hour maturation *in vitro*.

Table-60: Optimization of *in vitro* drug sensitivity assay against *P. knowlesi* : Haematocrit* versus maturation of parasite

Per cent Haematocrit	Hours	Mean Count/200 parasites ± S.E.			Mean Percent Parasitaemia±SE
		Ring	Trophozoite	Schizont	
3	0	200	0	0	0.9
	22	0	8±1.9	192±1.9	1±0.01
6	0	200	0	0	0.9
	22	0	35±2.2	165±2.2	0.9±0.04
12	0	200	0	0	0.9
	22	0	86±2.8	114±2.8	1.1±0.05
18	0	200	0	0	0.9
	22	30±0.98	85±7.5	85±6.5	1.0±0.08

*in 200 µl volume of media.

Table-61: Optimization of *in vitro* drug sensitivity assay against *P. knowlesi* : Volume of medium* versus maturation of parasite

Volume of medium (µl)	Hours	Mean Count/200 parasites ± S.E.			Mean Percent Parasitaemia±SE
		Ring	Trophozoite	Schizont	
50	0	200	–	–	0.9
	22	0	18±0.94	182±0.94	1±0.07
100	0	200	–	–	0.9
	22	0	15±1.1	185±1.1	1±0.04
200	0	200	–	–	0.9
	22	0	28±3.6	172±3.6	1.1±0.07
300	0	200	–	–	0.9
	22	13±1.4	49±5.6	138±4.6	1.0±0.05

*at 6% haematocrit.

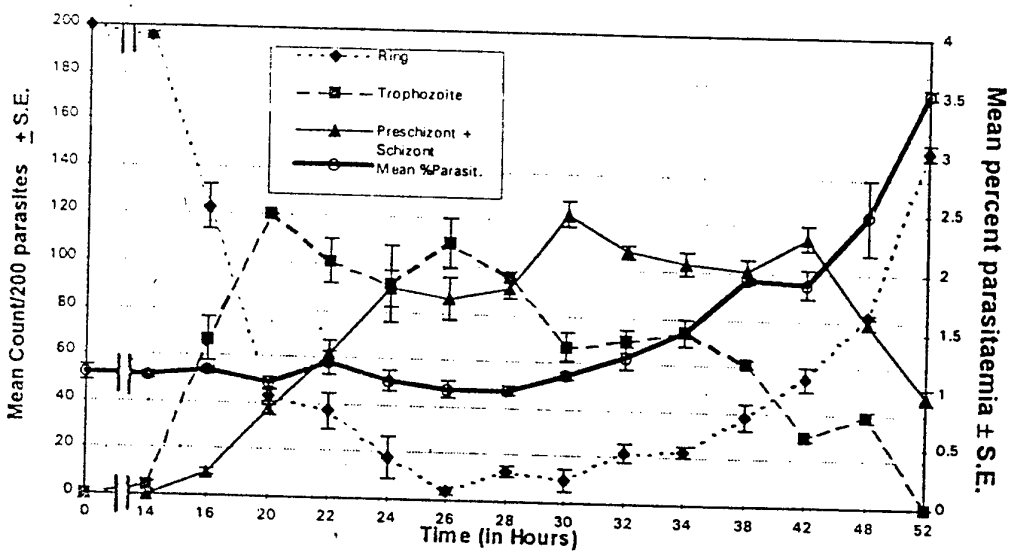
Table- 62: Optimization of *in vitro* drug sensitivity assay against *P. knowlesi* : Initial parasitaemia* versus maturation of parasite

Initial Parasitaemia (percentage)	Hours	Mean Count/200 parasites ± S.E.			Mean Percent Parasitaemia±SE
		Ring	Trophozoite	Schizont	
7.0	0	200	–	–	7.0
	22	130±5.9	38±4.5	32±1.2	6.9±0.16
3.9	0	200	–	–	3.9
	22	24±2.1	81±1.44	95±2.9	3.9±0.15
1.0	0	200	–	–	1.0
	22	2±0.82	51±4.2	147±4.9	1.0±0.07
0.3	0	200	–	–	0.3
	22	0	11±1.7	189±1.7	0.3±0.03

*in 200 µl of media at 6% haematocrit.

Table-63: Optimization of *in vitro* drug sensitivity assay against *P. falciparum*: Differential count of sorbitol synchronized intraerythrocytic stages during 52 hour duration

Hours	Mean count/200 parasites ± S.E.						Mean Percent parasitaemia ± S.E.
	Ring		Trophozoite		Pre Schizont	Schizont	
	Early	Late	Early	Late			
							
0	148 ± 2.6	52 ± 2.6	0	0	0	0	1.04 ± 0.07
14	151 ± 0.47	45 ± 1.9	4 ± 1.5	0	0	0	1.03 ± 0.03
16	67 ± 4.7	57 ± 14.7	56 ± 6.8	10 ± 3.9	7 ± 1.1	2 ± 2.2	1.07 ± 0.03
20	2 ± 1.1	41 ± 1.1	100 ± 3.3	20 ± 3.2	29 ± 2.2	8 ± 0	0.98 ± 0.05
22	3 ± 2.2	35 ± 9.5	77 ± 2.2	24 ± 7.5	37 ± 5.8	24 ± 3.8	1.15 ± 0.10
24	11 ± 6.3	7 ± 3.1	64 ± 13.6	28 ± 8.4	38 ± 4.9	51 ± 12.4	1.01 ± 0.09
26	0	4 ± 1.7	84 ± 11.3	26 ± 0.94	32 ± 3.8	54 ± 5.6	0.95 ± 0.07
28	0	13 ± 2.3	86 ± 2.3	11 ± 1.1	32 ± 0.54	58 ± 4.6	0.95 ± 0.04
30	0	10 ± 4.9	44 ± 8.2	22 ± 2.4	34 ± 4.9	90 ± 0.94	1.09 ± 0.03
32	22 ± 3.9	0	51 ± 3.4	19 ± 1.4	26 ± 1.9	82 ± 1.2	1.25 ± 0.1
34	23 ± 2.6	0	63 ± 4.1	11 ± 1.6	32 ± 1.1	71 ± 4.9	1.48 ± 0.12
38	38 ± 5.6	0	48 ± 2.5	13 ± 1.4	25 ± 1.9	76 ± 2.6	1.93 ± 0.03
42	55 ± 5.4	0	24 ± 0.72	6 ± 0.72	32 ± 0.94	83 ± 4.7	1.91 ± 0.12
48	82 ± 0.54	0	35 ± 2.2	4 ± 1.5	41 ± 1.25	38 ± 2.2	2.48 ± 0.31
52	152 ± 3.54	0	0	0	24 ± 3.27	24 ± 1.91	3.53 ± 0.04



Differential count of sorbital synchronized *P. falciparum* intraerythrocytic stages during 52 hour maturation *in vitro*.

Table-64 : Comparison of uptake of different radioactive precursors in in vitro culture of P. cynomolgi and p.knowlesi using 6% haematocrit.

S.No.	Radiolabelled precursors	P.knowlesi(5-6%)	P.cynomolgi (2-3%)
1.	^{3}H Thymidine (0.5 µCi/well)	1348±278.50	1119.33±197.30
2.	^{3}H Leucine (1µCi/well)	12034±350.39	6190±272.42
3.	^{3}H Isoleucine (0.5µCi/well)	1979±181.5	4711.66±268.07
4.	^{3}H Hypoxanthine (0.5 µCi/well)	25769±3255.66	17404±733.33

Table-65 : Determination of optimum concentration of ^{3}H hypoxanthine during the *in vitro* growth of *P.knowlesi*.

	Percent parasitaemia	Radioactive uptake (DPM) 0.5 µCi	0.25 µCi	0.125 µCi
Expt.I	2.5%	16476.66±2675.25	9307-20±1047.60	6815.00±108.25
Expt.II	11%	23230±3140.0	9556.0±1248.80	-
	4%	25769.0±3255.0	14026.0±2076.0	-
	1%	17017.0±445.0	12321.0±992.0	-
	NRBC	606.00±84.85	343.00±32.52	-

Table-66: Incorporation of ^{3}H hypoxanthine during short term *in vitro* culture of *P.knowlesi* ;

Effect of duration of incubation on uptake .

Culture condition		Hypoxanthine uptake counts (DPM) at	
Parasitaemia	haematocrit	4 hrs	24 hrs
EXPERIMENT-1			
9 %	6%	3806.66±628..0	14546.66±1375.80
	3%	2504.0±98.66	28562.6±1264.65
	115%	1228.6±86.93	34707.0±3559.75
	0.75%	796.33±96.77	37017.66±3698.97
3%	6%	1318.66±79.32	24236.0±2373.0
	3%	945.0±71.92	23573.0±1656.31
	1.5%	787.0±172.86	26473.3±1207.83
	0.75%	540.33±51.39	13263.3±1307.16
1.0%	6%	837.0±296.6	19223.6±2090.4
	3%	547.00±84.48	16954.0±542.87
	1.5%	565.66±104.40	8931.0±1541.16
	0.75%	507.50±61.51	3892.0±336.99
NRBC	6%	390.5±47.37	586.0±137.16
	3%	477.66±56.88	467.66±242.73
	1.5%	895.33±31.89	444.0±157.70
EXPERIMENT-II			
8%	6%	1602.33±319.64	17611.00±2509.40
	1.5%	818.00±2.82	28441.66±7884.83
3%	6%	621.33±102.88	15136.66±1486.89
	1.5%	373.66±24.84	4508.50±624.37
0.18%	6%	466.00±158.39	4600.00±1127.58
	1.5%	346.00±86.11	776.50±369.81
NRBC	6%	480.00±73.13	573.66±95.55
	1.5%	329.50±13.43	521.33±117.73

Table-67: Incorporation of ^{3}H-hypoxanthine during short term *in vitro* culture of *P.knowlesi*:

Effect of varible parasitaemia and haematocrit on the uptake.

Parasitaemia	Radioactive incorporation (DPM)			
	6%	3%	1.5%	0.75%
EXPERIMENT-1				
10%	14546.66±1375.8	28562.6±1264.65	34707.0±3559.75	37817.66±3698.9
3%	24236.0±2373.0	23573.0±1656.31	26473.3±1207.83	13267.3±1307.16
1%	19223.6±2090.4	16954.0±542.87	8931.0±1541.16	3892.0±336.99
NRBC	586.0±137.16	467.66±242.73	444.0±137.70	437.66±195.19
EXPERIMENT-II				
8%	17611.00±2509.40		28441.66±3280.40	
3%	15136.66±1486.89		4508.50±624.37	
0.8%	4600.00±1127.58		776.50±3699.81	
NRBC	573.66±95.55		521.33±117.73	

Table-68: Evaluation of dose response of chloroquine during short term in vitro culture of P.knowlesi.

Chloroquine concentration (ug/ml)	Exp.1	Exp.2.	Exp.3.
10.00	622	587	1067
2.50	1354	707	988
.625	1041	527	896
.156	663	684	1063
.0390	1042	736	17823
.0097	1124	19877	22828
.0024	18086	28346	24209
.00060	21482	27084	26032
.00015	20426	26448	26031
Control	20581	30628	26996
IC 50	0.0236	0.036	0.0366
95% limit	(0.017-0.030)	(0.029-0.044)	(0.0283-0.0474)
IC 90	0.299	0.373	0.6371
95% limit	(0.211-0.421)	(0.272-0.513)	(0.4258-0.9532)

Table -69 : **Viability of *P. falciparum* post chloroquine exposure *in vitro***

Hours of Drug Exposure	Concentration (ng/ml)	GROWTH IN TERMS OF % PARASITAEMIA AFTER CHLOROQUINE EXPOSURE ± S.D.											
		48 hours				96 hours				144 hours			
		Ring	Tropho-zoite	Schizont	Total	Ring	Tropho-zoite	Schizont	Total	Ring	Tropho-zoite	Schizont	Total
Control		3.1± 0.13	0.03± 0.02	0.39± 0.24	*3.5± 0.35*	6.7± 0.69	0.76± 0.2	1.52± 0.64	*8.9± 0.15*	5.4± 0.48	2.7± 0.42	2.2± 0.39	*10.4± 0.41*
3	200	2.3± 0.14	0.13± 0.02	0.43± 0.14	*2.8± 0.24*	4.4± 0.28	1.18± 0.24	1.63± 0.21	*7.2± 0.29*	5.3± 0.26	3.03± 0.33	2.97± 0.09	*11.4± 0.25*
	1000	1.43± 0.17	0.09± 0.09	0.4± 0.14	*1.94± 0.18*	4.6± 0.46	0.97± 0.33	1.53± 0.38	*7.1± 0.33*	4.77± 0.45	2.13± 0.26	1.87± 0.21	*8.8± 0.57*
	5000	0.64± 0.08	0.18± 0.02	0.05± 0.04	*0.87± 0.09*	0.79± 0.21	1.2± 0.08	0.18± 0.14	*2.2± 0.09*	6.5± 0.52	1.37± 0.21	0.64± 0.09	*8.5± 0.28*
6	200	1.28± 0.21	0.07± 0.06	0.32± 0.05	*1.67± 0.28*	3.8± 0.34	0.49± 0.15	1.6± 0.05	*5.8± 0.48*	5.9± 0.71	2.3± 0.39	2.5± 0.85	*10.7± 1.6*
	1000	0.43± 0.22	0.25± 0.04	0.37± 0.02	*1.05± 0.22*	1.82± 0.09	1.27± 0.12	1.37± 0.26	*4.5± 0.17*	5.1± 0.52	2.9± 0.34	1.31± 0.39	*9.2± 0.49*
	5000	0.63± 0.15	0.21± 0.05	0	*0.84± 0.17*	0.91± 0.21	0.92± 0.13	0.2± 0.18	*1.9± 0.28*	4.0± 0.69	0.53± 0.23	0.27± 0.22	*4.8± 1.1*
12	200	0.59± 0.25	0.1± 0.05	0.31± 0.07	*1.0± 0.37*	2.95± 0.05	0.37± 0.08	0.89± 0.11	*4.2± 0.26*	5.5± 0.22	1.8± 0.34	2.1± 0.42	*9.4± 0.83*
	1000	0.51± 0.06	0.11± 0.02	0.07± 0.01	*0.7± 0.06*	0.78± 0.27	0.56± 0.12	0.54± 0.12	*1.9± 0.28*	4.2± 0.54	1.43± 0.65	0.53± 0.17	*6.2± 0.16*
	5000	0.59± 0.03	0.15± 0.02	0	*0.74± 0.02*	0.89± 0.04	0.52± 0.31	0.19± 0.06	*1.79± 0.07*	4.9± 0.62	1.24± 0.37	0.29± 0.08	*6.6± 0.12*
24	200	0.71± 0.11	0.04± 0.03	0.03± 0.02	*0.77± 0.11*	2.6± 0.27	0.12± 0.05	0.34± 0.03	*3.04± 0.25*	4.5± 0.2	1.12± 0.18	1.6± 0.05	*7.2± 0.1*
	1000	0.54± 0.06	0.04± 0.03	0	*0.58± 0.09*	0.59± 0.03	0	0.02± 0.03	*0.61± 0.03*	2.1± 0.8	0.21± 0.14	0.18± 0.01	*2.51± 0.69*
	5000	0.6± 0.05	0.03± 0.02	0	*0.63± 0.03*	0.43± 0.05	0.11± 0.04	0.06± 0.04	*0.59± 0.05*	1.48± 0.38	0.18± 0.09	0.16± 0.09	*1.8± 0.36*
36	200	0.41± 0.009	0	0	*0.41± 0.09*	0.12± 0.01	0.01± 0.01	0	*0.13± 0.02*	0.65± 0.02	0.12± 0.02	0.07± 0.02	*0.83± 0.03*
	1000	0.37± 0.03	0	0	*0.37± 0.03*	0.1± 0	0.01± 0.01	0	*0.11± 0.01*	0.22± 0.06	0.05± 0	0.06± 0.01	*0.33± 0.07*
	5000	0.49± 0.1	0	0	*0.49± 0.1*	0.22± 0.03	0.01± 0.01	0	*0.23± 0.03*	0.08± 0.02	0.05± 0	0.03± 0	*0.15± 0.02*
48	200	0.41± 0.11	0	0	*0.41± 0.11*	0.02± 0	0	0	*0.02± 0*	0.08± 0.01	0.03± 0.004	0.01± 0.009	*0.12± 0.01*
	1000	0.52± 0.04	0	0	*0.52± 0.04*	0.02± 0.01	0	0	*0.02± 0.01*	0.05± 0.02	0	0	*0.05± 0.02*
	5000	0.61± 0.05	0	0	*0.61± 0.05*	0.06± 0.02	0	0	*0.06± 0.02*	0.02± 0.01	0	0	*0.02± 0.01*
60	200	0.29± 0.09	0	0	*0.29± 0.09*	0.06± 0.02	0	0	*0.06± 0.02*	0.03± 0	0	0	*0.03± 0*
	1000	0.41± 0.07	0	0	*0.41± 0.07*	0.08 ± 0.01	0	0	*0.08 ± 0.01*	0.04± 0.009	0	0	*0.04± 0.009*
	5000	0.12± 0.08	0	0	*0.12± 0.08*	0.04± 0.02	0	0	*0.04± 0.02*	0.04± 0.02	0	0	*0.04± 0.02*
72	200	0.04± 0.02	0	0	*0.04± 0.02*	0.04± 0.009	0	0	*0.04± 0.009*	0	0	0	*0*
	1000	0.08± 0.01	0	0	*0.08± 0.01*	0.03± 0.01	0	0	*0.03± 0.01*	0	0	0	*0*
	5000	0.1± 0.04	0	0	*0.1± 0.04*	0.03± 0.01	0	0	*0.03± 0.01*	0	0	0	*0*

Delay in appearance of viable parasites is indicated by shaded area.

Table-70: Viability of *P. falciparum* after mefloquine exposure in vitro

Hours of Drug Exposure	Concentration (ng/ml)	GROWTH IN TERMS OF % PARASITAEMIA AFTER MEFLOQUINE EXPOSURE ± S.D.											
		48 hours				96 hours				144 hours			
		Ring	Trophozoite	Schizont	Total	Ring	Trophozoite	Schizont	Total	Ring	Trophozoite	Schizont	Total
Controls	-	3.1 ± 0.13	0.03 ± 0.02	0.39 ± 0.29	*3.5 ± 0.35*	6.7 ± 0.69	0.76 ± 0.2	1.52 ± 0.64	*8.9 ± 0.15*	5.4 ± 0.98	2.7 ± 0.4	2.2 ± 0.29	*10.4 ± 0.41*
3	200	0.54 ± 0.06	0.4 ± 0.06	0.04 ± 0.03	*0.99 ± 0.06*	0.41 ± 0.13	0.49 ± 0.18	0.05 ± 0.04	*0.94 ± 0.27*	1.93 ± 0.63	0.55 ± 0.11	0.04 ± 0.06	*2.52 ± 0.69*
	1000	0.75 ± 0.13	0.04 ± 0.03	0	*0.78 ± 0.11*	0.5 ± 0.05	0	0	*0.5 ± 0.05*	0.33 ± 0.05	0	0	*0.33 ± 0.05*
	5000	0.74 ± 0.13	0.05 ± 0.05	0	*0.79 ± 0.09*	0.49 ± 0.07	0	0	*0.49 ± 0.07*	0.16 ± 0.02	0	0	*0.16 ± 0.02*
6	200	0.69 ± 0.2	0.2 ± 0.10	0.02 ± 0.02	*0.91 ± 0.11*	0.68 ± 0.06	0.25 ± 0.07	0	*0.93 ± 0.12*	1.04 ± 0.11	0.04 ± 0.05	0.03 ± 0.04	*1.11 ± 0.07*
	1000	0.85 ± 0.15	0.03 ± 0.04	0	*0.88 ± 0.17*	0.58 ± 0.03	0.02 ± 0.02	0	*0.59 ± 0.04*	0.33 ± 0	0	0	*0.33 ± 0*
	5000	0.61 ± 0.03	0.07 ± 0.02	0	*0.68 ± 0.04*	0.51 ± 0.07	0	0	*0.51 ± 0.07*	0.16 ± 0.07	0	0	*0.16 ± 0.07*
12	200	0.72 ± 0.16	0.19 ± 0.05	0.02 ± 0.02	*0.91 ± 0.10*	0.67 ± 0.10	0.36 ± 0.13	0.05 ± 0.04	*1.07 ± 0.17*	1.8 ± 0.26	0.24 ± 0.12	0	*2.1 ± 0.17*
	1000	0.81 ± 0.05	0.06 ± 0.02	0	*0.87 ± 0.07*	0.57 ± 0.05	0	0	*0.57 ± 0.05*	0.14 ± 0.03	0	0	*0.14 ± 0.03*
	5000	0.66 ± 0.009	0.12 ± 0.05	0	*0.77 ± 0.04*	0.45 ± 0.02	0	0	*0.45 ± 0.02*	0.08 ± 0.005	0	0	*0.08 ± 0.005*
24	200	0.72 ± 0.02	0	0	*0.72 ± 0.02*	0.37 ± 0.04	0	0	*0.37 ± 0.04*	0.09 ± 0.02	0	0	*0.09 ± 0.02*
	1000	0.66 ± 0.06	0	0	*0.66 ± 0.06*	0.14 ± 0.02	0	0	*0.14 ± 0.02*	0.07 ± 0.03	0	0	*0.07 ± 0.03*
	5000	0.59 ± 0.04	0.06 ± 0.02	0	*0.63 ± 0.07*	0.19 ± 0.03	0	0	*0.19 ± 0.03*	0.07 ± 0.02	0	0	*0.07 ± 0.02*
36	200	0.78 ± 0.13	0	0	*0.78 ± 0.01*	0.21 ± 0.01	0	0	*0.21 ± 0.01*	0.09 ± 0.09	0	0	*0.9 ± 0.09*
	1000	0.67 ± 0.07	0	0	*0.67 ± 0.07*	0.13 ± 0.03	0	0	*0.13 ± 0.03*	0.08 ± 0.01	0	0	*0.08 ± 0.01*
	5000	0.5 ± 0.06	0	0	*0.51 ± 0.06*	0.13 ± 0.03	0	0	*0.13 ± 0.03*	0.06 ± 0.009	0	0	*0.06 ± 0.009*
48	200	0.64 ± 0.05	0	0	*0.64 ± 0.05*	0.12 ± 0	0	0	*0.12 ± 0*	0.08 ± 0.01	0	0	*0.08 ± 0.01*
	1000	0.46 ± 0.09	0	0	*0.46 ± 0.09*	0.08 ± 0.03	0	0	*0.08 ± 0.03*	0.07 ± 0	0	0	*0.07 ± 0*
	5000	0.44 ± 0.12	0	0	*0.44 ± 0.12*	0.05 ± 0.12	0	0	*0.05 ± 0.12*	0.04 ± 0.009	0	0	*0.04 ± 0.009*
60	200	0.69 ± 0.12	0	0	*0.69 ± 0.12*	0.07 ± 0.01	0	0	*0.07 ± 0.01*	0.06 ± 0.009	0	0	*0.06 ± 0.009*
	1000	0.47 ± 0.08	0	0	*0.47 ± 0.08*	0.06 ± 0.01	0	0	*0.06 ± 0.01*	0.06 ± 0.009	0	0	*0.06 ± 0.009*
	5000	0.56 ± 0.06	0	0	*0.56 ± 0.06*	0.05 ± 0.02	0	0	*0.05 ± 0.02*	0.05 ± 0.02	0	0	*0.05 ± 0.02*
72	200	0.19 ± 0.06	0	0	*0.19 ± 0.06*	0	0	0	*0*	0	0	0	*0*
	1000	0.07 ± 0.03	0	0	*0.07 ± 0.03*	0	0	0	*0*	0	0	0	*0*
	5000	0.07 ± 0	0	0	*0.07 ± 0*	0	0	0	*0*	0	0	0	*0*

Table-71 : Bioassay of chloroquine activity and chloroquine equivalents against *P. knowlesi* in sequential serum samples from chloroquine treated rhesus monkeys

Monkey No.	Dose mg/kg	Hours after drug administration																									
		0	1		2		4		6		12		24		30		36		48		72		96		120		144	
		MI	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE
101	10	0	-	-	100	313	-	-	100	313	100	313	100	313	100	157	-	-	-	-	-	-	-	-	-	-	-	-
102	10	0	-	-	100	313	-	-	100	313	100	626	100	626	-	-	100	626	100	313	100	313	100	157	100	157	-	-
103	10	0	100	313	100	313	-	-	100	626	100	626	100	626	-	-	100	626	100	313	100	313	100	313	100	157	100	157
104	20	0	-	-	100	1252	100	1252	100	1252	100	1252	100	1252	100	1252	100	1252	-	-	-	-	-	-	-	-	-	-
105	30	0	-	-	100	1252	100	1252	100	2504	100	2504	100	1252	100	1252	-	-	-	-	-	-	-	-	-	-	-	-
106	30	0	100	626	100	1252	-	-	100	1252	100	1252	100	1252	-	-	100	2504	100	1252	100	1252	100	1252	100	626	100	626

MI = Percent inhibition schizont maturation in presence of serum samples (1/2 dilution)

DE = Drug (Chloroquine) equivalent (ng/ml) in serum samples (1/2 dilution)

\- = Not done

Table-72: : Bioassay of halofantrine activity and halofantrine equivalents against *P. knowlesi* in sequential serum samples from halofantrine treated rhesus monkeys

Monkey No.	Dose mg/kg	Hours after drug administration																					
		0	2		4		6		12		24		30		36		48		72		96		120	
		MI	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE	MI	DE
301	10	0	100	125	-	-	100	125	100	125	100	125	100	125	-	-	-	-	-	-	-	-	-	-
302	10	0	100	63	-	-	100	63	100	63	100	63	-	-	100	63	100	63	69	-	28	-	17	-
303	20	0	100	500	100	500	100	500	100	125	100	125	100	125	100	63	-	-	-	-	-	-	-	-
304	30	0	100	250	100	1000	100	1000	100	1000	100	500	100	500	-	-	-	-	-	-	-	-	-	-
305	30	0	100	250	-	-	100	250	100	250	100	250	-	-	-	-	100	250	100	125	100	63	100	25

MI = Percent inhibition schizont maturation in presence of serum samples (1/2 dilution)
DE = Drug (halofantrine) equivalent (ng/ml) in serum samples (1/2 dilution)
\- = Not done

Table-73: In vitro methemoglobin estimation using mastomys erythrolysate as a source of hemoglobin (substrate).

S.No.	Additives	Molar concentrations	Hemolysate concentrations	Range of % of Met Hb
1.	Control(PBS)	Not applicable	20%	Nil
2.	$NaNO_2$	10 uM		5.1=8.6%
		100 uM	"	18-22.7%
		1000 uM		80-89%
		2.5 mM		100%
3.	Chloroquine	10^{-3} M		2.0-3.7%
		10^{-6} M		Nil
		10^{-9} M		Nil
4.	Primaquine	10 uM		3.0-4.5%
		100 uM		8.0-11.6%
		1000 uM (10^{-3} M)		23.8-29.6%
5.	4 mPQ	10^{-3} M		41.8-50%

table-74: :Prophylactic activity of rHU-IL-12 against challenge with sporozoites of P.cynomolgi B.

Group	Dose	No.of doses	Treatment days	Monkey no.	Result	Cure rate
1	100ng/kg	7	(-2 to+10)*	1	Patency on day 11	0/4
				2	Patency on day 11	
				3	Patency on day 12	
				4	Patency on day 11	
2.	1 ug/kg	7	(-2 to +10)*	1	Patency on day 13	0/4
				2	Patency on day 12	
				3	Patency on day 18	
				4	Patency on day 13	
3.	10 ug/kg	1	(-2)	1	Cured	4/4
				2	Cured	
				3	Cured	
				4	Cured	
4.	20 ug/kg	2	(-2 and 0)	1	Cured	2/2
				2	Cured	
5.	Nil	Nil		1.	Patency on day 10	0/4
				2	Patency on day 10	
				3	Patency on day 10	
				4	Patency on day 12	

*Treatment on alternate days

Table-75: Prophylactic activity rHu-IL-12 (revalidation) against challenge with sporozoites of P.cynomolgi B.

Group	Dose	No.of doses	Treatment days	Monkey No.	Result	Cure rate
1	10ug/kg	1	(-2)	1	Cured	3/3*
				2	Cured	
				3	Cured	
2.	Control	Nil			Patency on day 10	Nil

Table-76: Cytokine mRNA expression in controls, the groups that received multiple doses of rHuIL-12 (Grp 1 and 2), and the protected group that received a single dose (Grp 3) on the day of peak mRNA expression in Grp 3 for the 6 cytokines for which there was a significant elevation in monkeys that received rHuIL-12 as compared to controls.

(Assay carried out at CDC)

Cytokine	Day of Peak in Grp 3	Geometric Mean No. of Transcripts[a]			
		Contr Grp	Grp 1 100 ng/kg multiple doses	Grp 2 1 µg/kg multiple doses	Grp 3 10 µg/kg single dose
IL-6	2	1.8	1.0	2.4	26.0*
IL-10	2	7.7	23.2	31.4	82.2**
IL12α	0	1.0	1.0	2.1	11.4*
IL-15	0	1.0	1.0	1.0	8.5*
IFN-γ	4	2.3	24.5*	111.2[b]*	1809*
TNF-α	0***	13	12	59	71*

[a] Geometric mean transcripts on day of peak in the 10 µg/kg, single dose group (Grp3). Day of peak expression of mRNA was the same for all groups except for IFN-γ in Grp2 as noted below.

[b] Peak for this group was on day 2; the geometric mean number of transcripts on day 2 was 509.7.

* Significantly different ($p < 0.05$) from control (Mann-Whitney U test).

**p=0.059 for this group as compared to the control group by the Mann-Whitney U test. The p value for all other comparisons was > 0.10.

***Because TNF-α mRNA levels increased after sporozoite challenge in control and experimental groups (see text), we have only included data from monkeys prior to sporozoite challenge.

Table-77: Kinetics of cytokine mRNA expression in groups with significantly elevated levels: Fold Increase in mRNA expression over control monkey mRNA expression on same day.

		Day Relative to Sporozoite Challenge						
Cytokine	Group	Day -2	Day 0	Day 2	Day 4	Day 7	Day 11	Day 13
IL-6	3	1.0	1.0	14.6*	9.0	2.2	1.1	1.4
IL-10	3	1.0	4.0	10.6*	8.2	5.9	2.4	2.7
IL-12α	3	0.8	11.4*	2.6	0.6	1.2	1.8	1.2
IL-15	3	0.8	8.5*	1.9	1.6	1.0	1.0	1.0
IFNγ	1	0.8	6.4	24.2*	10.7*	1.1	1.8	2.2
	2	0.6	4.0	273.9*	48.8*	9.6	9.9	0.8
	3	0.8	23.0*	8.6*	792*	21.8*	7.7	3.9

* Number of transcipts in experimental group greater than in control monkeys ($p < 0.05$).

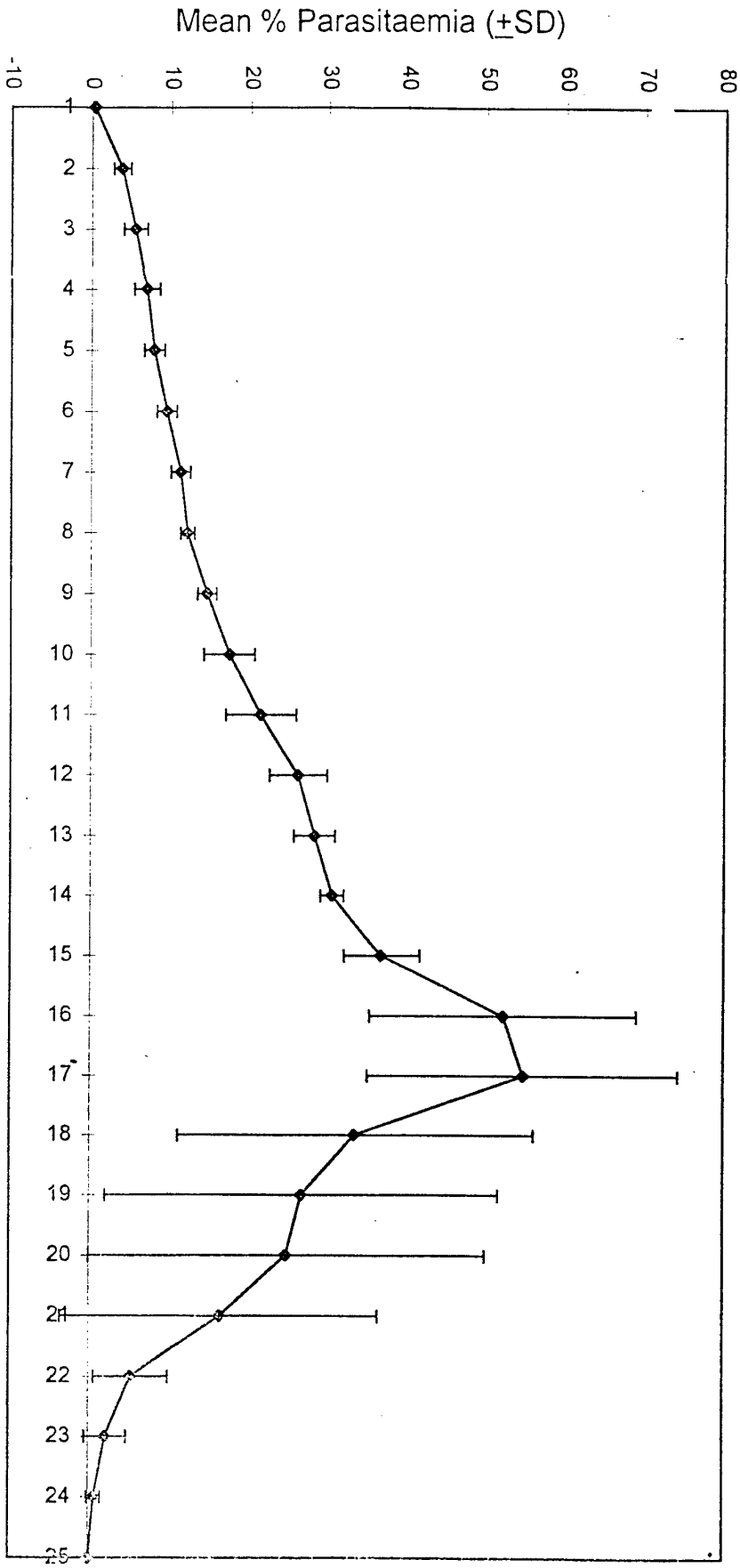


Fig. 1A: Course of P.yoelii nigeriensis infection in Golden hamsters

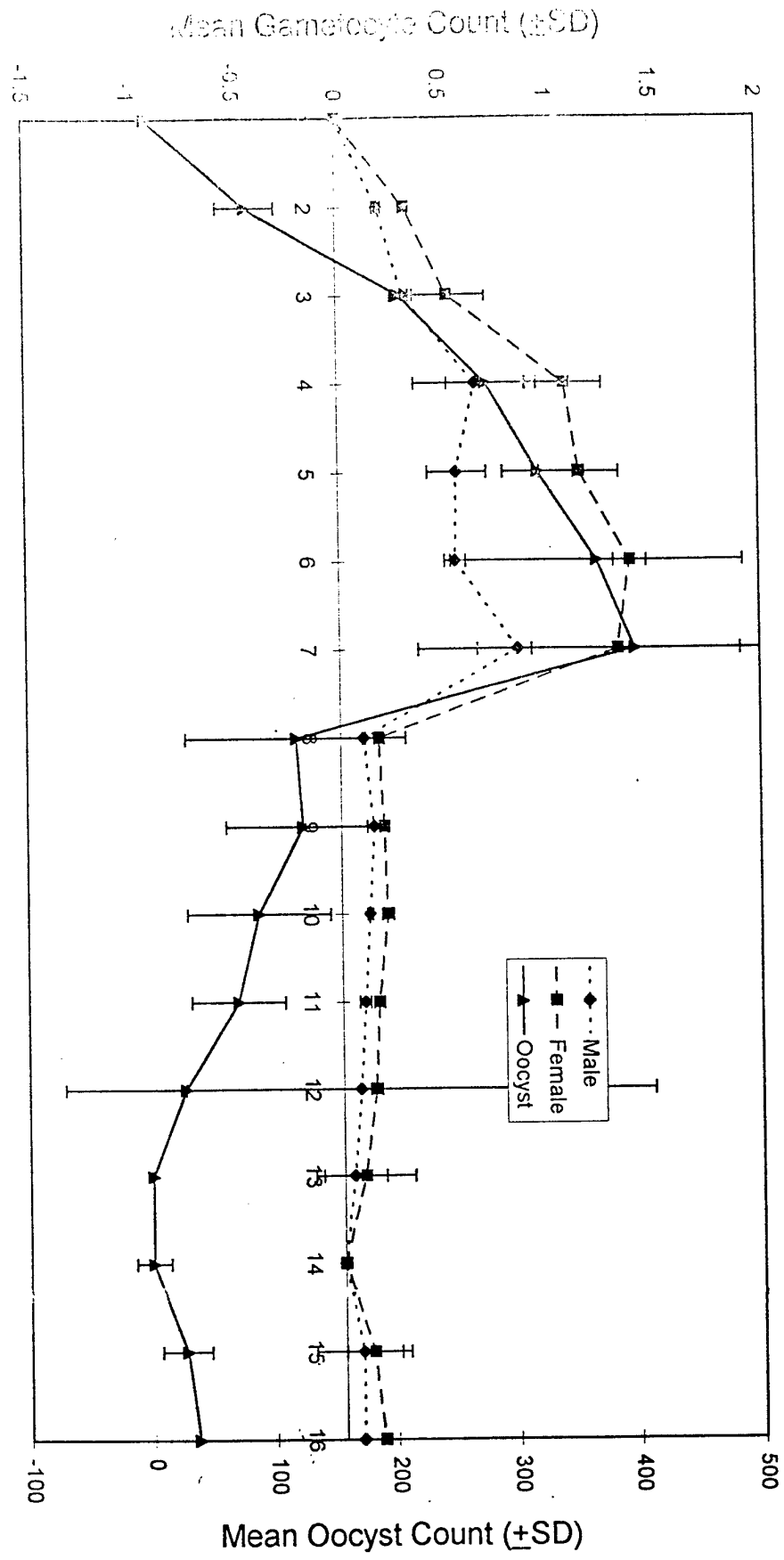


Fig 1B: P.yoelii nigeriensis **infection in Golden Hamsters: Correlation of gametocytaemia and infectivity to mosquitoes.**

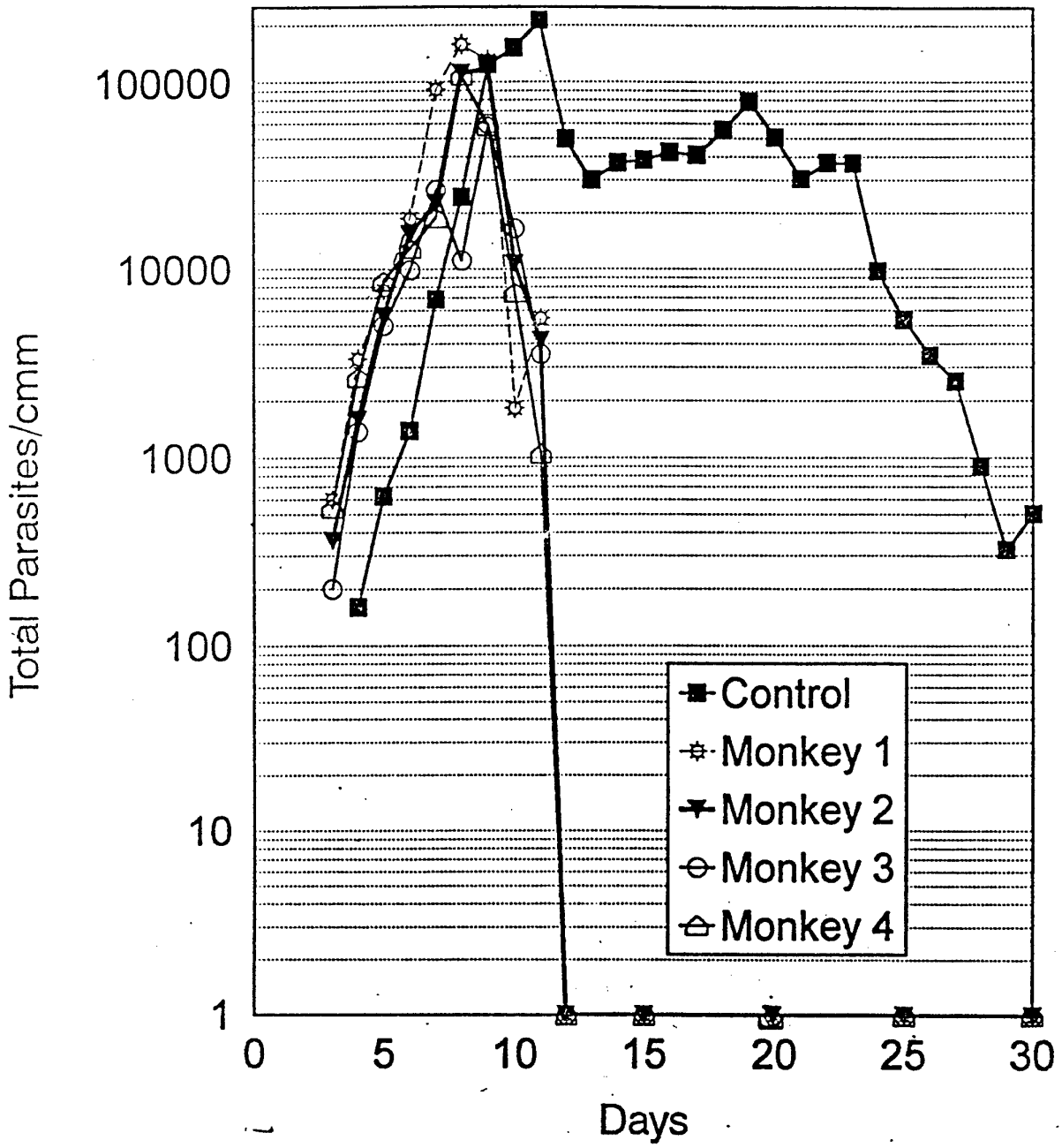


Fig.2. Blood schizontocidal response of Azithromycin against P.cyomolgi B in rhesus monkey.

Fig. 3

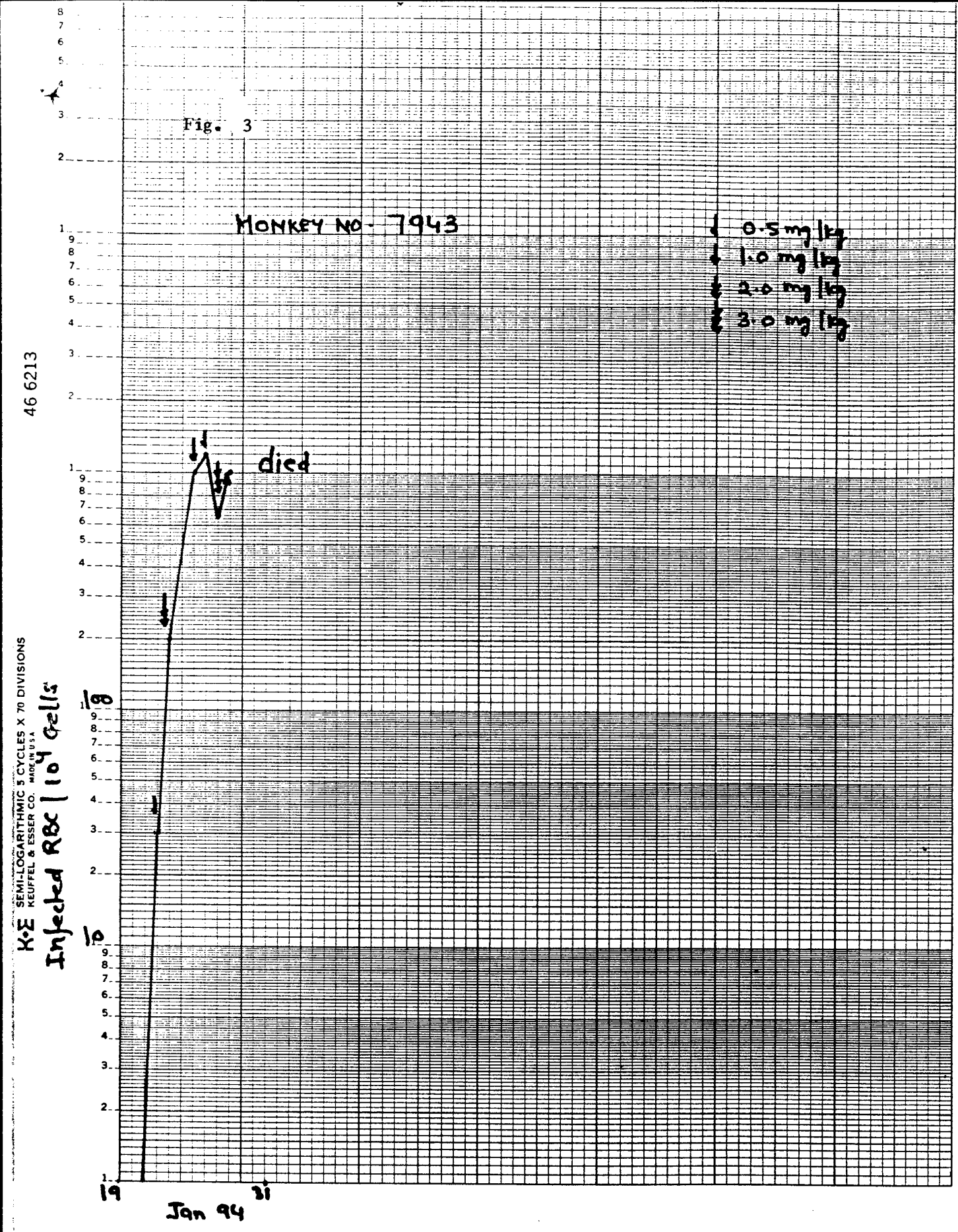

MONKEY NO - 7943
0.5 mg/kg
1.0 mg/kg
2.0 mg/kg
3.0 mg/kg
died
Injected RBC / 10⁴ Cells
1/100
1/10
19
31
Jan 94
46 6213
K·E SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.

Fig. 4

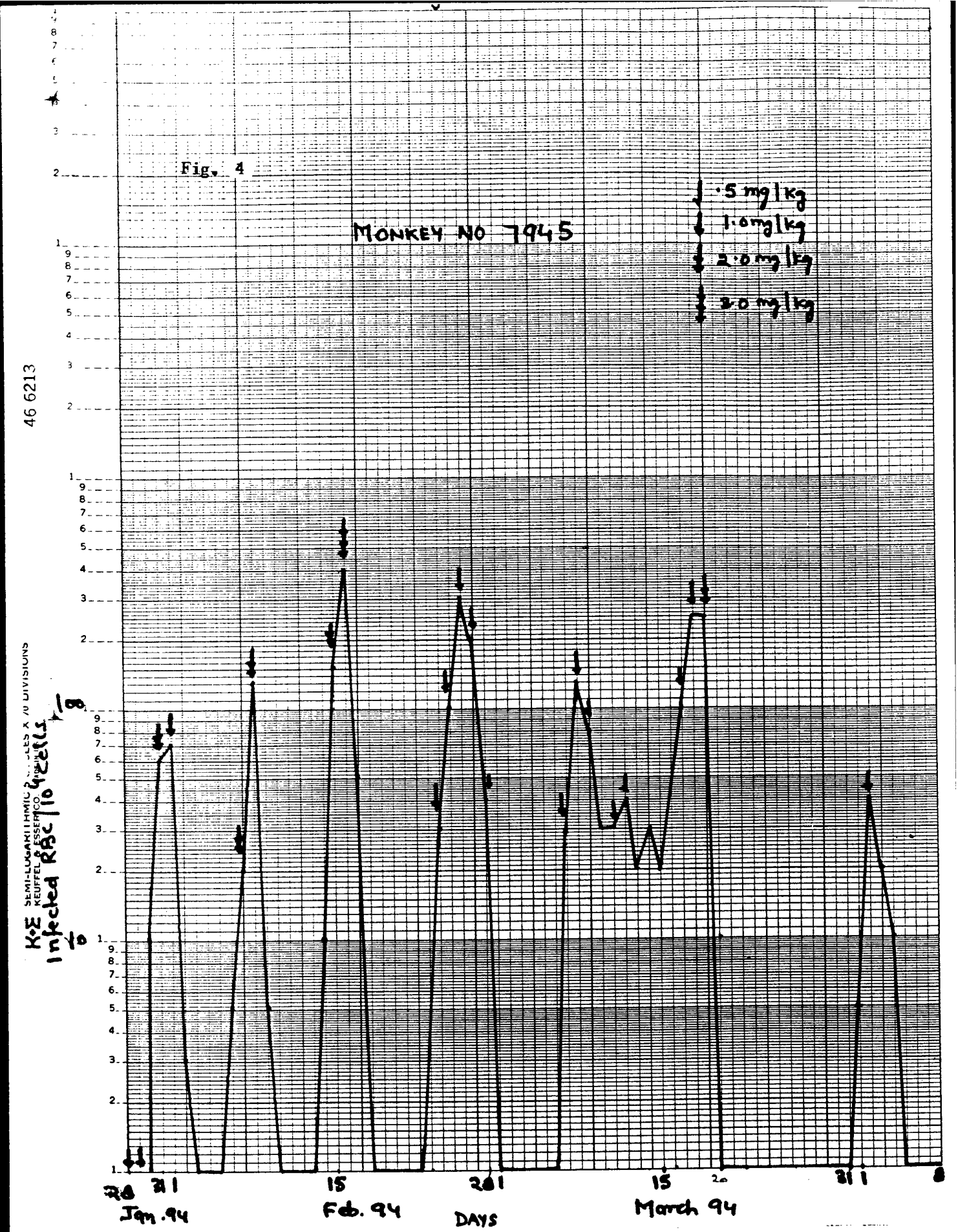
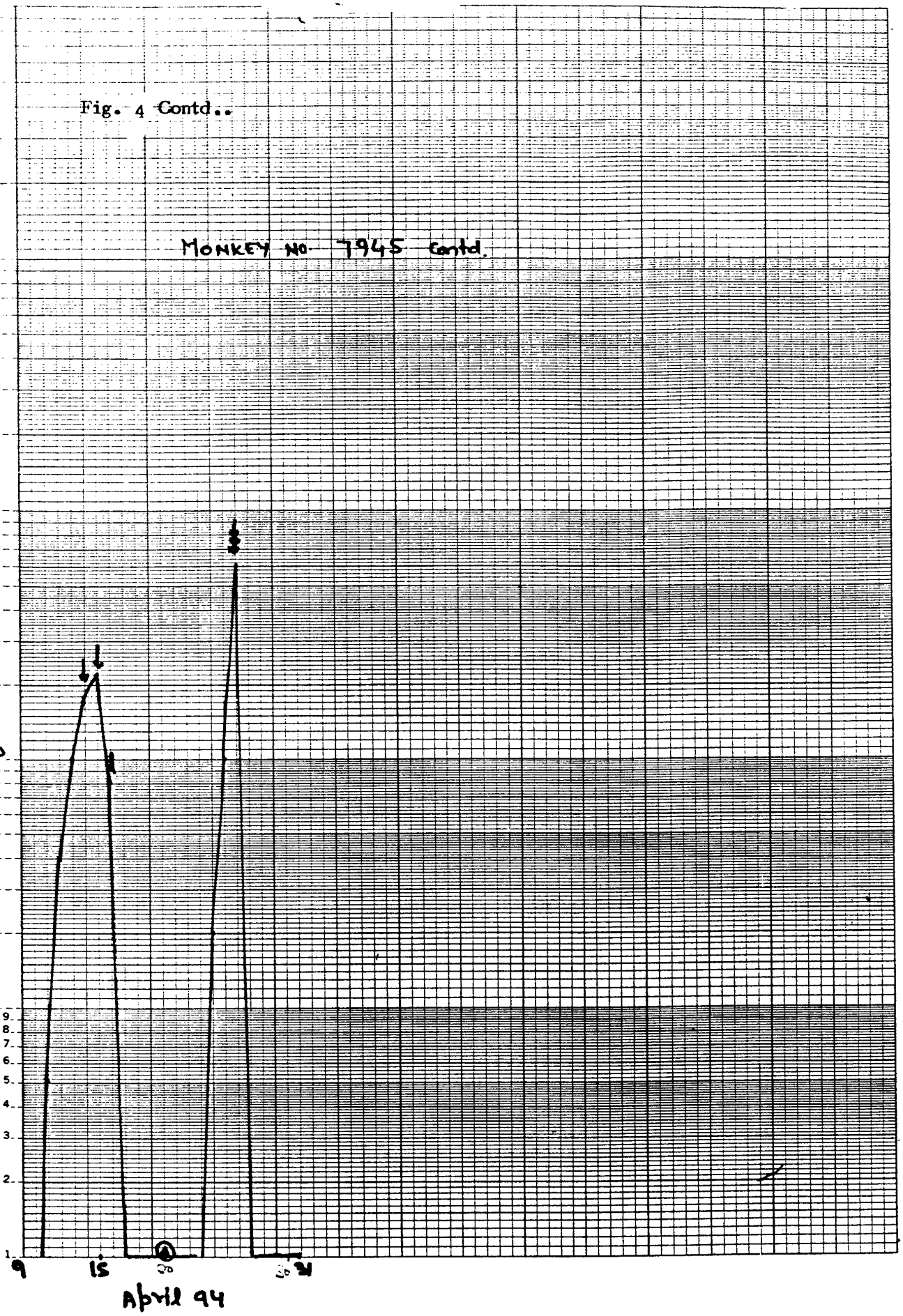

MONKEY NO 7945
·5 mg/kg
1.0 mg/kg
2.0 mg/kg
3.0 mg/kg
K.E Infected RBC/10 cells
DAYS
Jan. 94
Feb. 94
March 94

Fig. 4 Contd..



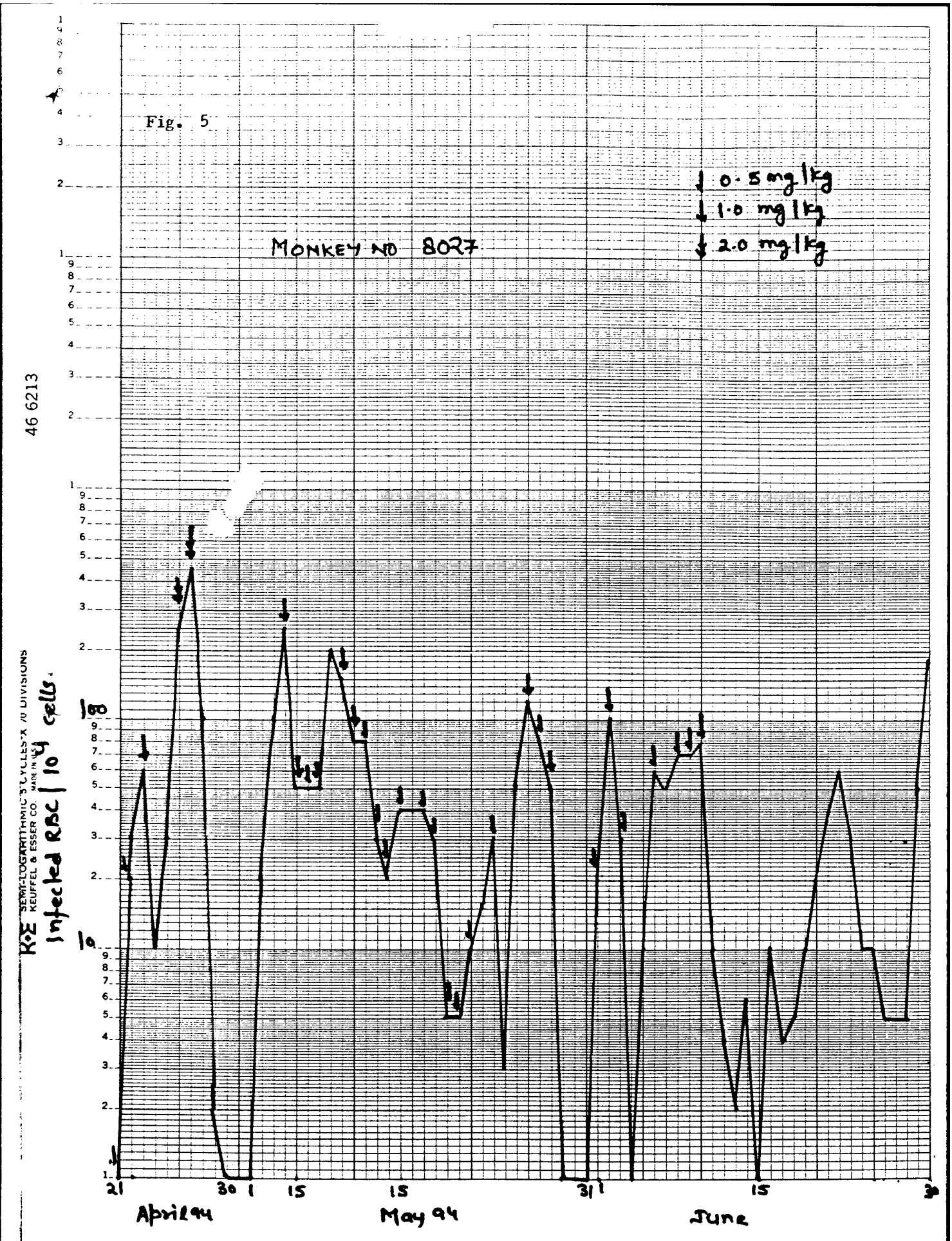


Fig. 5
MONKEY NO 8027
0.5 mg/kg
1.0 mg/kg
2.0 mg/kg
Infected RBC / 10⁴ cells
April 94
May 94
June
21
30
1
15
15
31
1
15
30

Fig. 5 (Contd.)

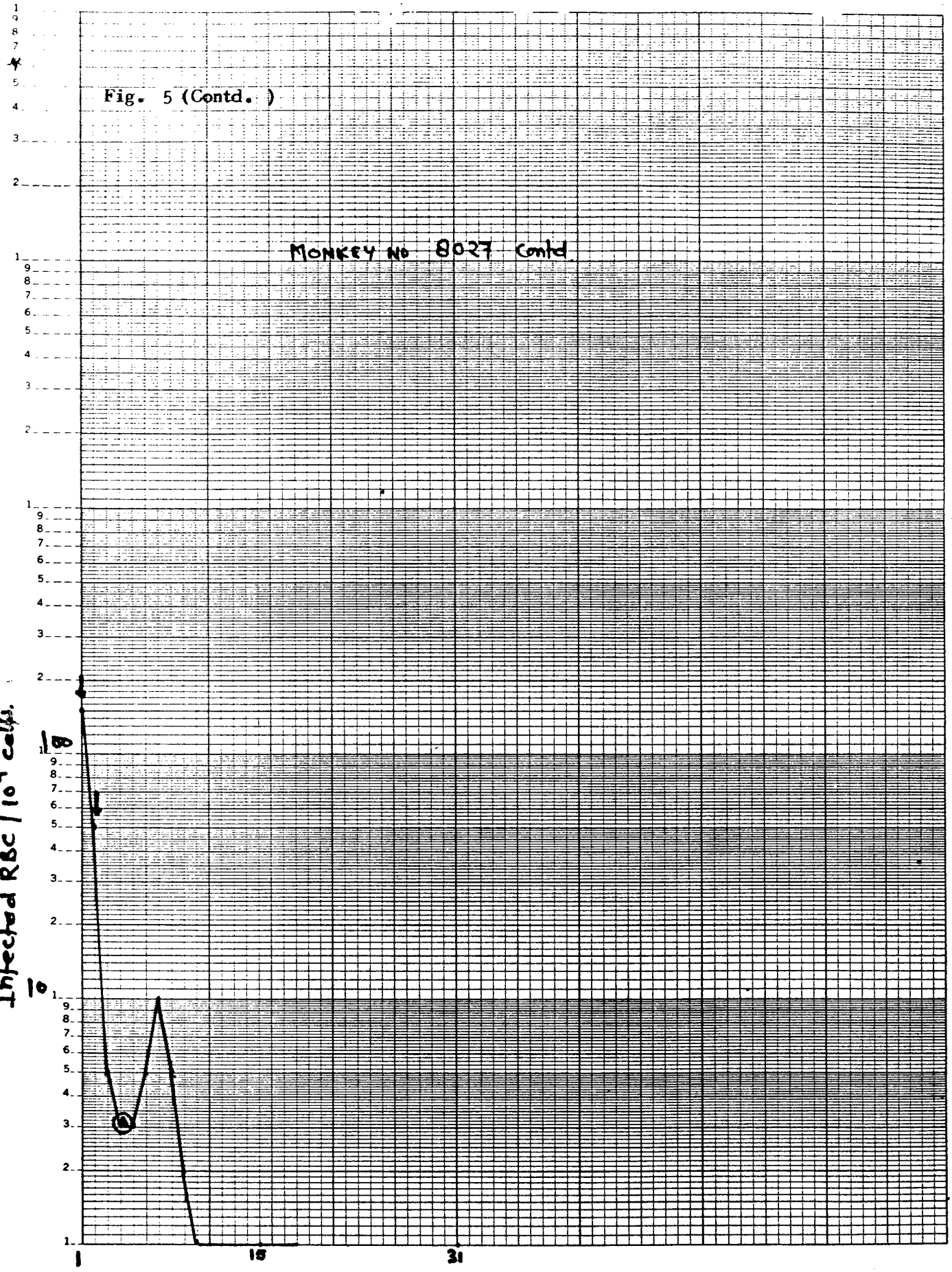


Fig. 6

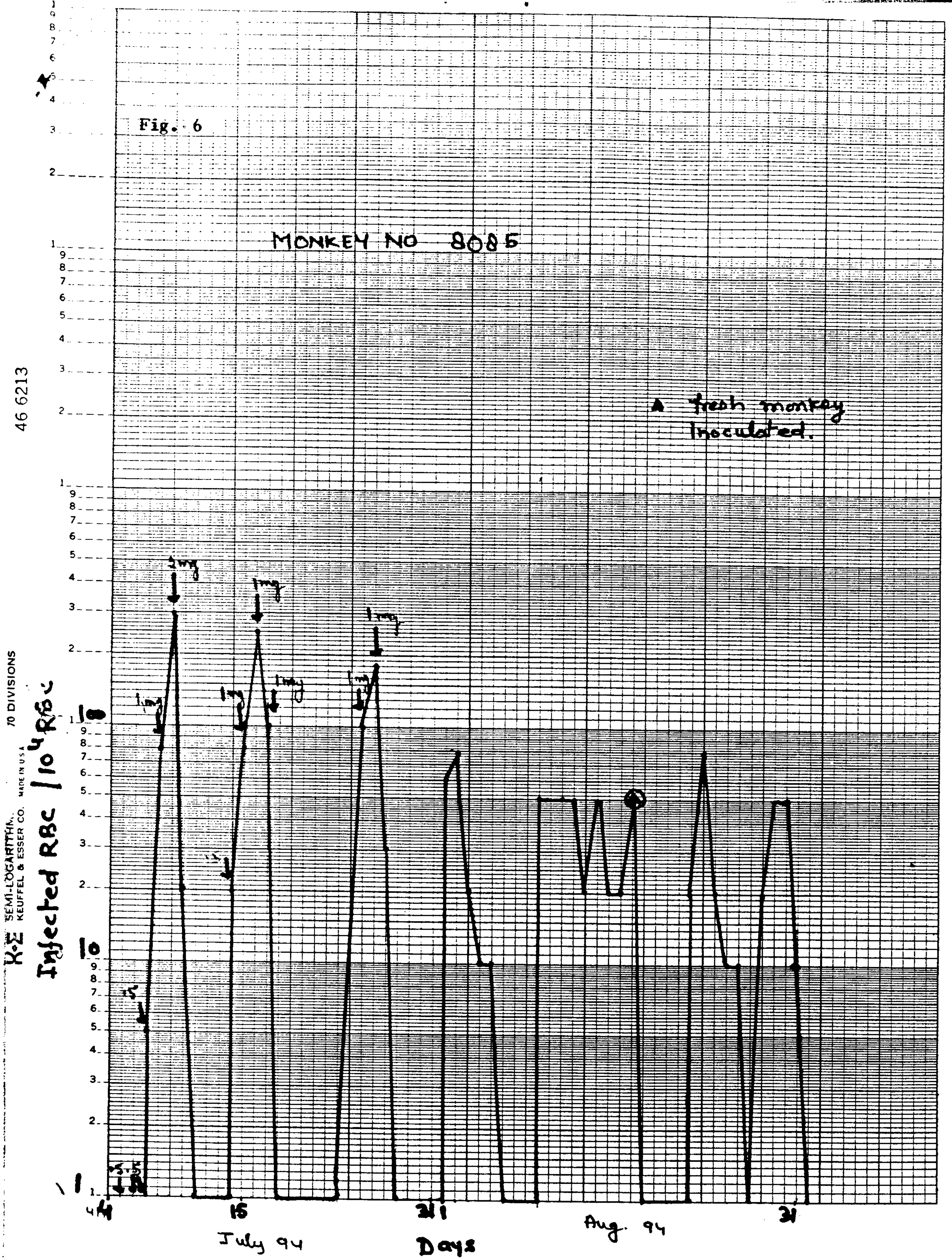
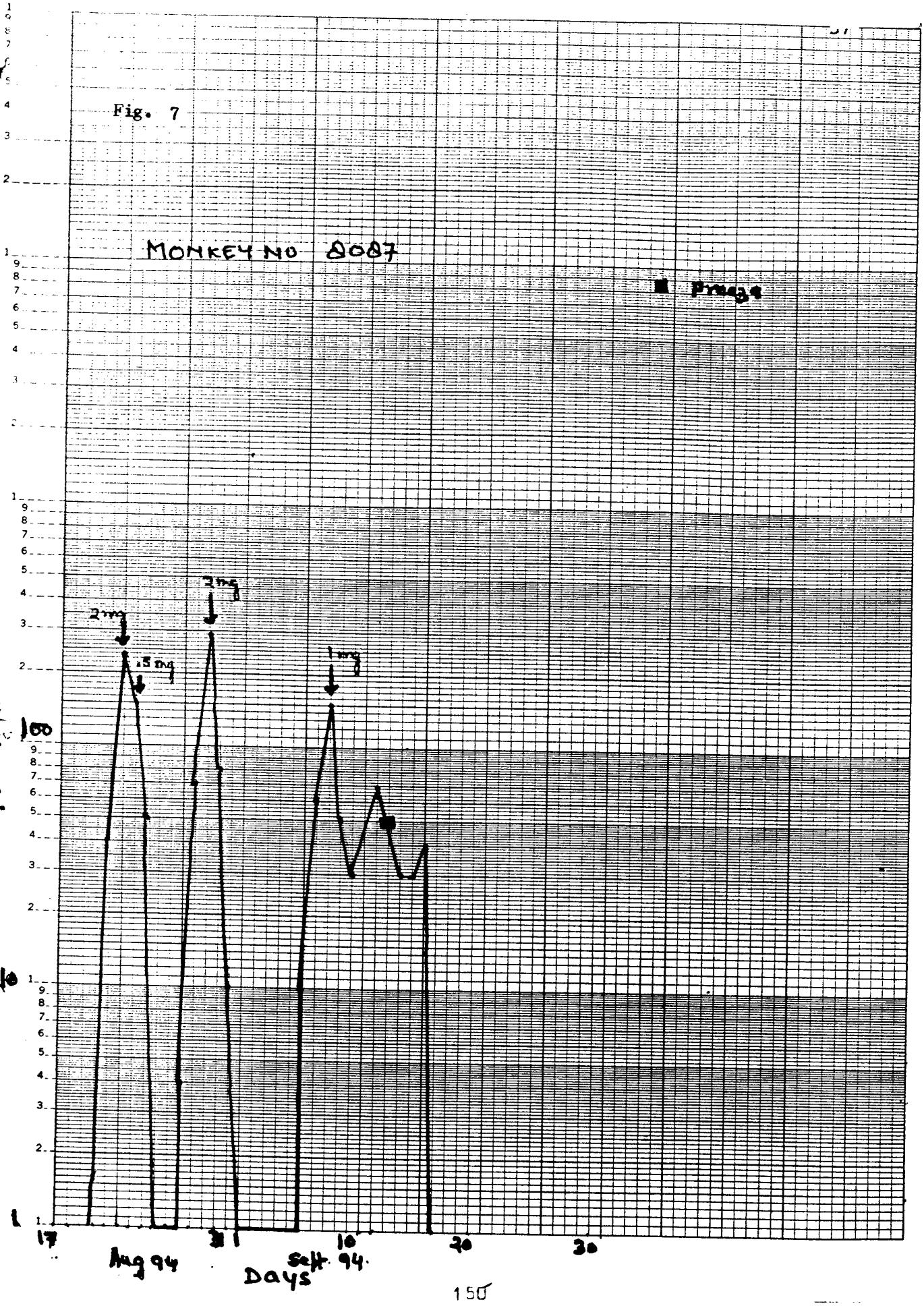

MONKEY NO 8085
▲ fresh monkey inoculated.
Infected RBC / 10⁴ RBC
Days
July 94
Aug. 94
1 mg
2 mg
.5
4
15
31
1
31
K+E SEMI-LOGARITHMIC
KEUFFEL & ESSER CO. MADE IN U.S.A.
70 DIVISIONS
46 6213

Fig. 7



SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
K·E 46 6213

Fig. 8

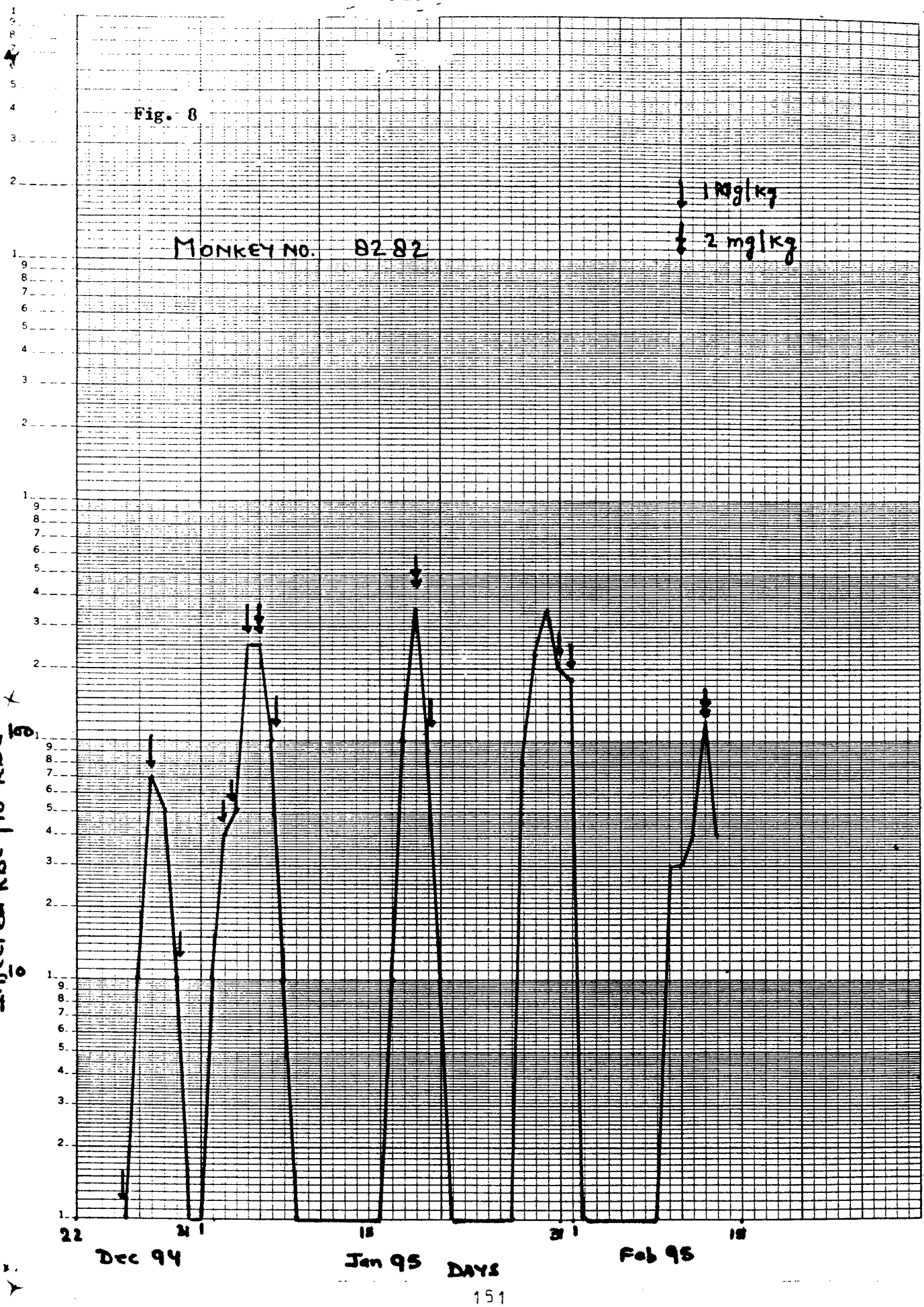


Fig. 9 Drug exposure of rhesus monkeys with mefloquine for selection of mefloquine resistant strain of P.knowlesi.

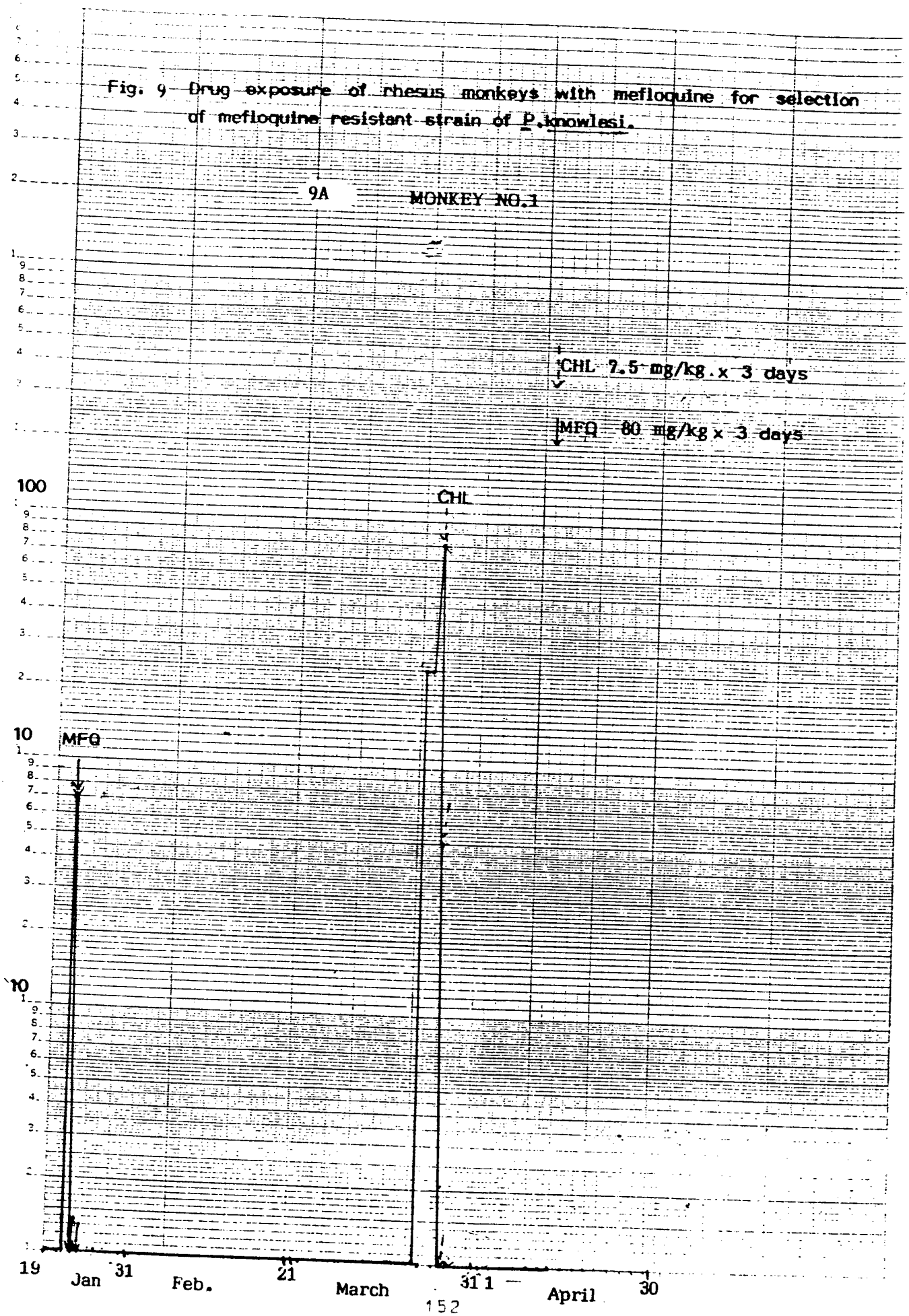


Fig. 5

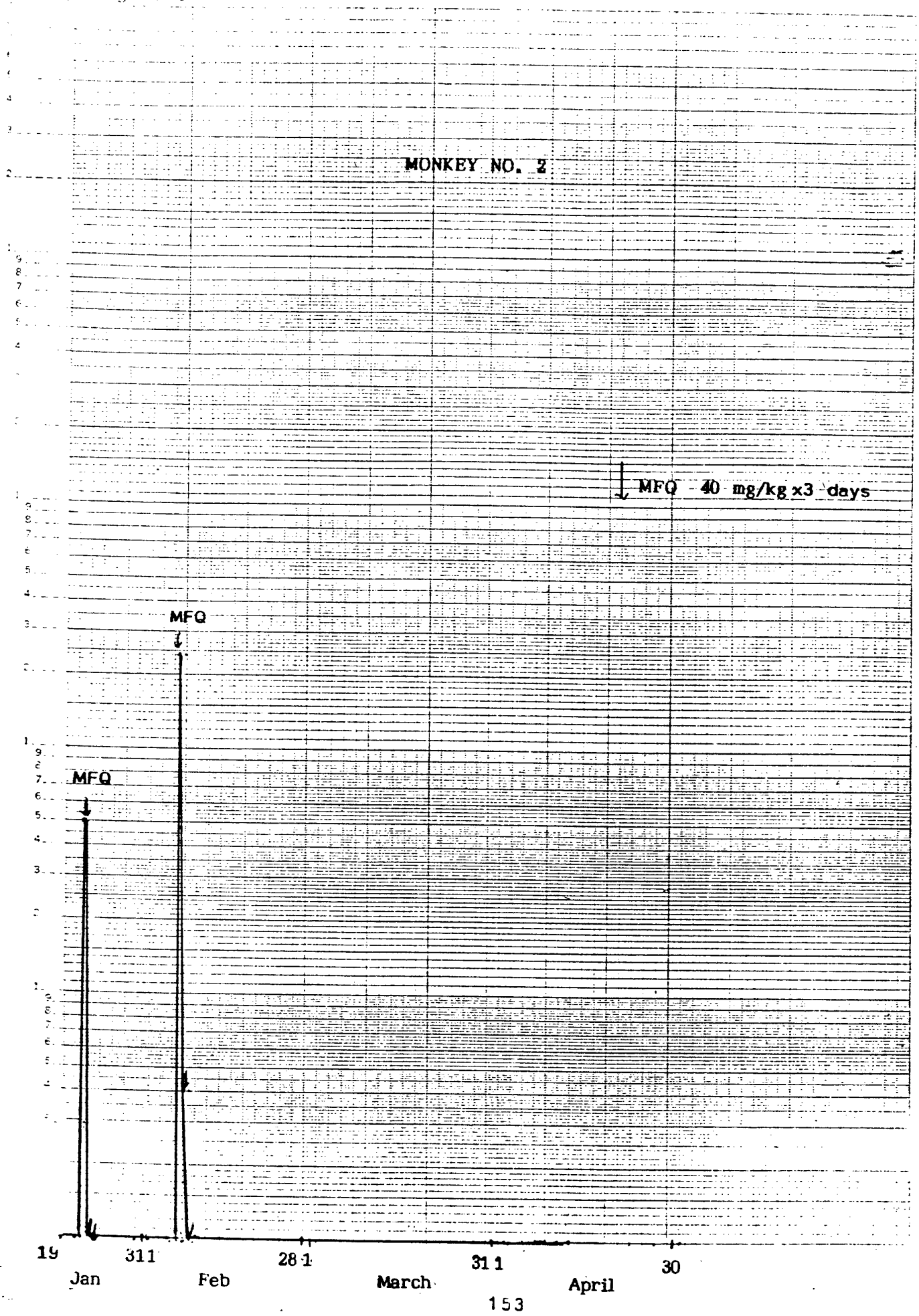

MONKEY NO. 2
MFQ 40 mg/kg x3 days
MFQ
MFQ
19
311
28 1
31 1
30
Jan
Feb
March
April

Fig. 9 C

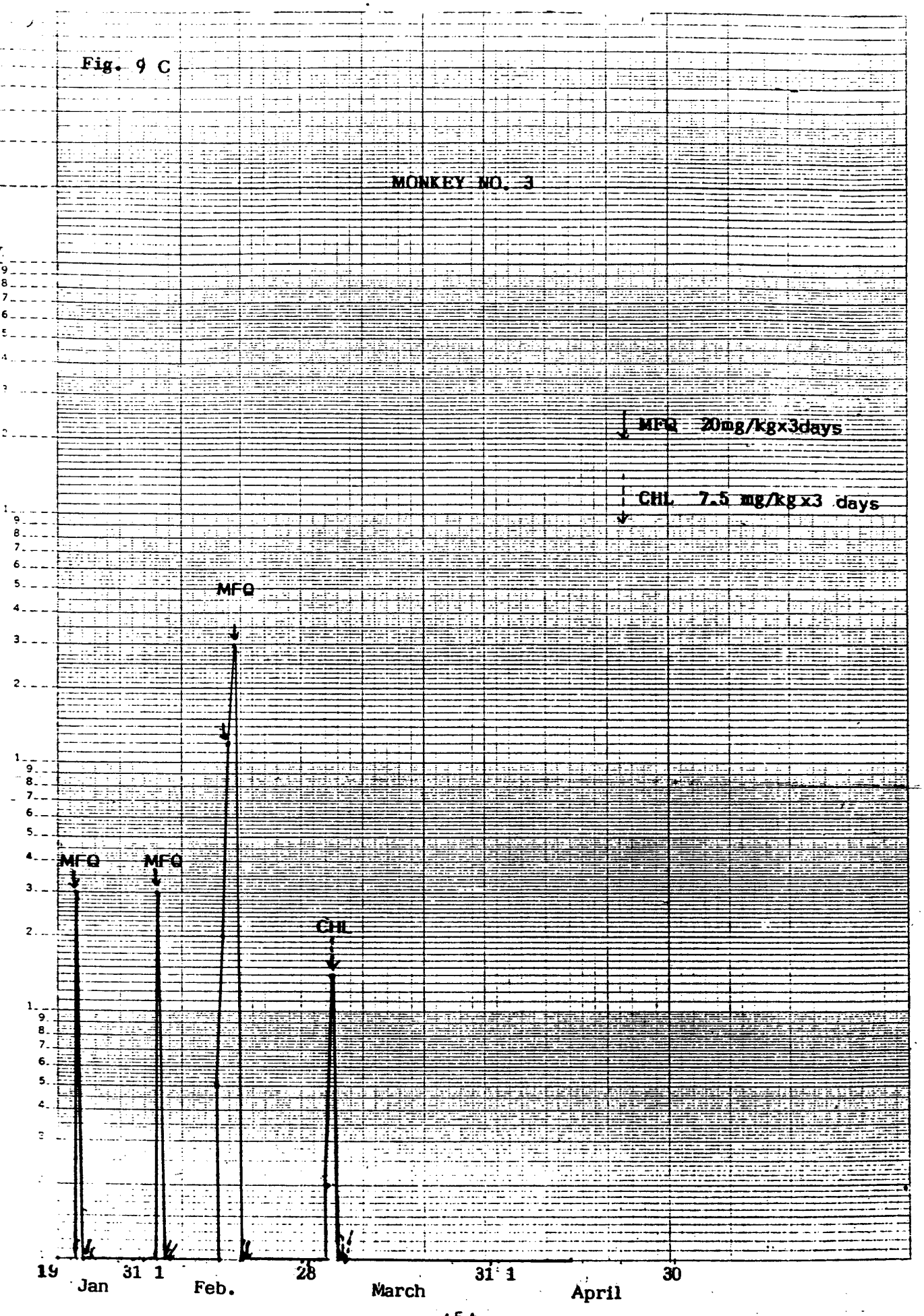

MONKEY NO. 3
MFQ 20mg/kg×3days
CHL 7.5 mg/kg×3 days
MFQ
MFQ
MFQ
CHL
19
31
1
28
31
1
30
Jan
Feb.
March
April

Fig. 10

P. knowlesi- Rhesus Monkey

Resistance Reversal Studies

Mefloquine+ Amitryptiline

Rx

MFQ = 20mg/kg x 3 days

ATT= 20mg/kg X 5 days

Monkey No.1 O.....O

2. Δ.....Δ

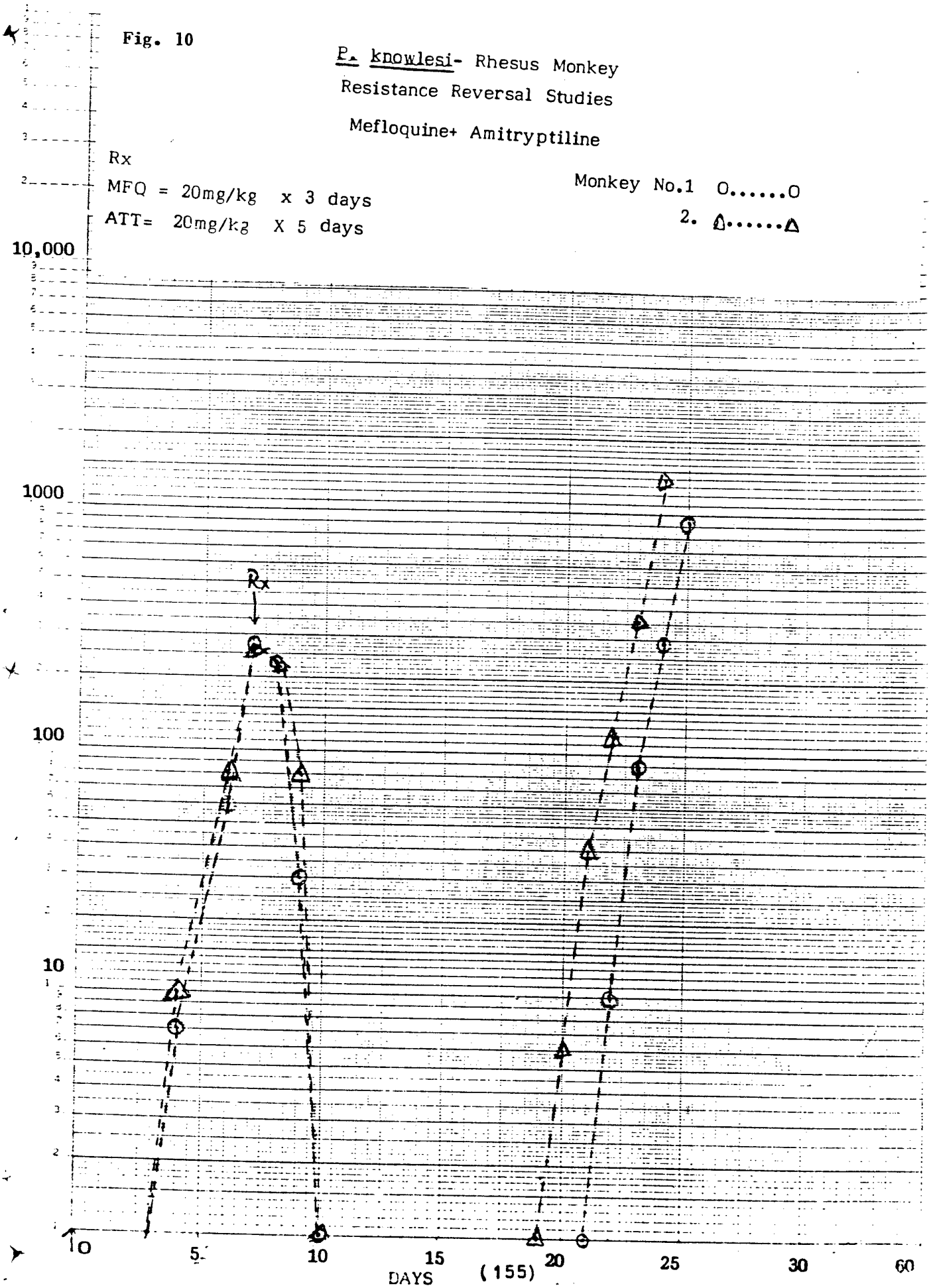
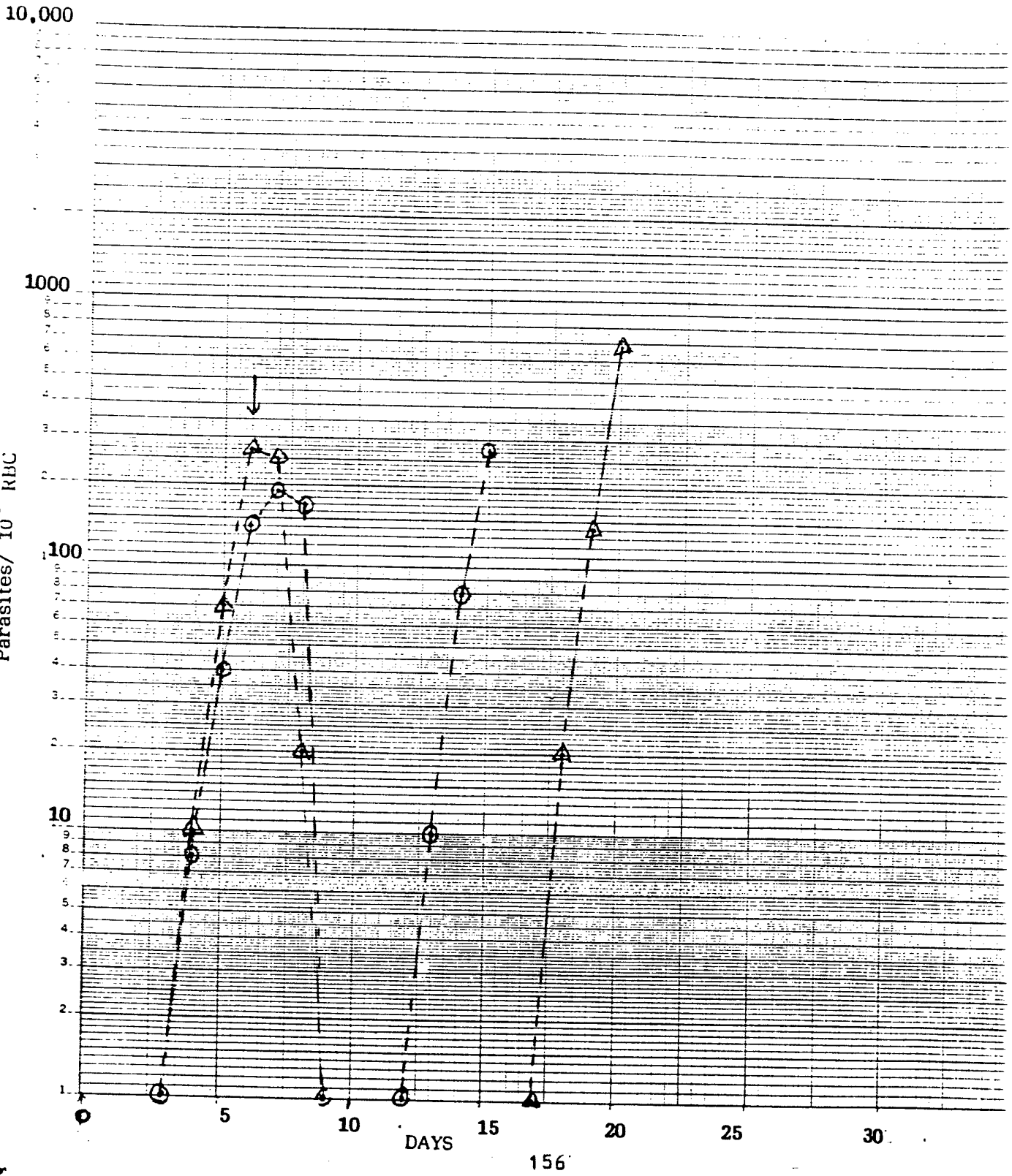


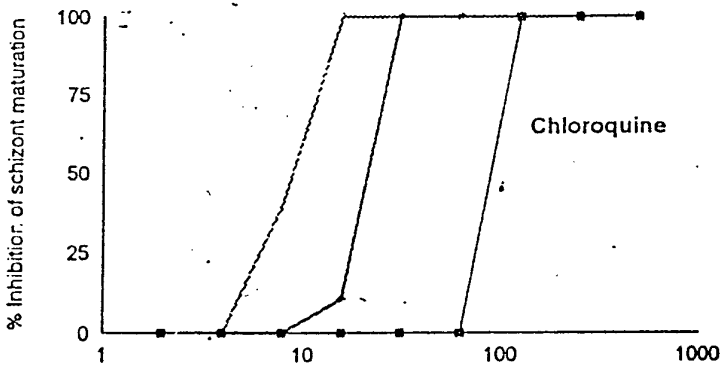
Fig. 11 P.knowlesi- Rhesus Monkey

Rx

Monkey No. 1. O.....O MFQ: 10mg/kgx3 days

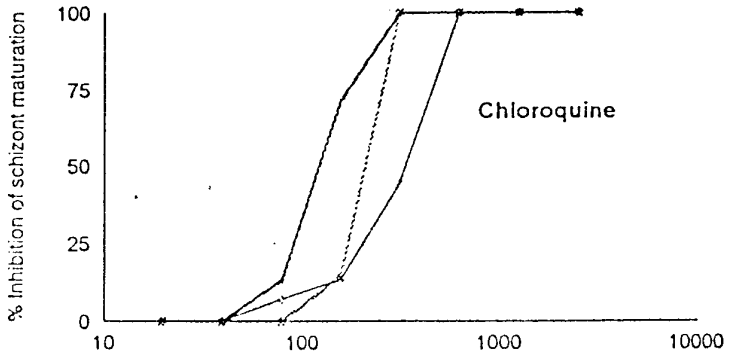
2. △.....△ MFQ: 20mg/kgx3 days





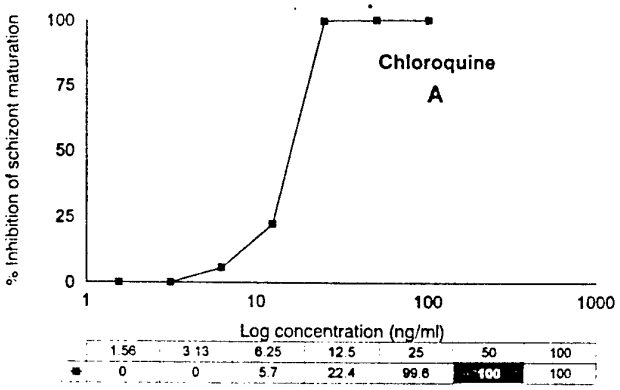
	1.95	3.9	7.8	15.6	31.3	62.5	125	250	500
11.1%	0	0	0	0	0	0	100	100	100
1.6%	0	0	0	10.5	100	100	100	100	100
0.23%	0	0	39.5	100	100	100	100	100	100

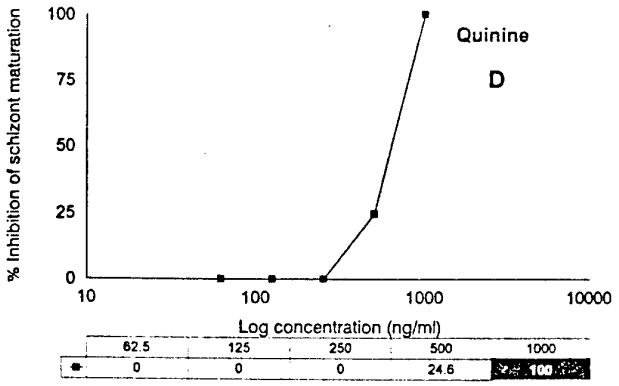
Fig.12A **Effect of initial parasitaemia on MIC of chloroquine against P.knowlesi in vitro**

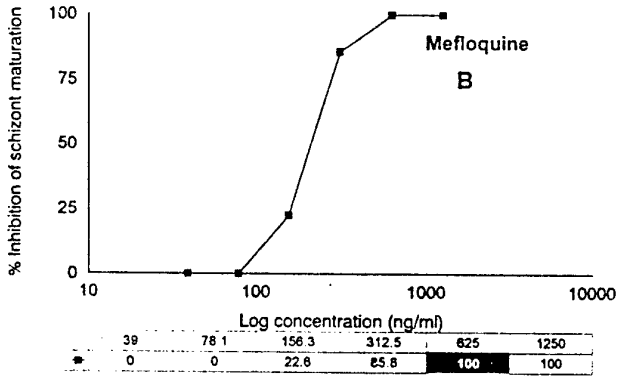


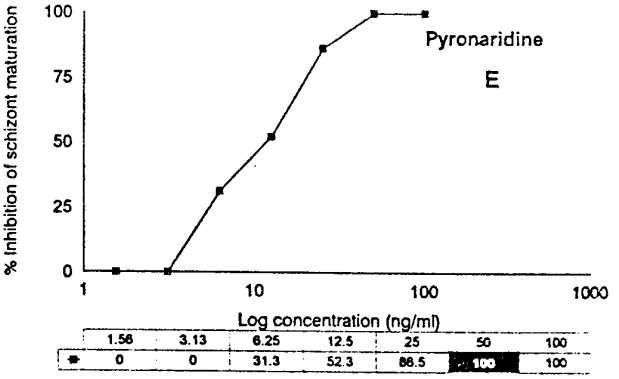
	19.5	39.06	78.13	156.3	312.5	625	1250	2500
4.7%	0	0	7	13.5	44.3	100	100	100
0.9%	0	0	13	71.8	100	100	100	100
0.12%	0	0	0	14.3	100	100	100	100

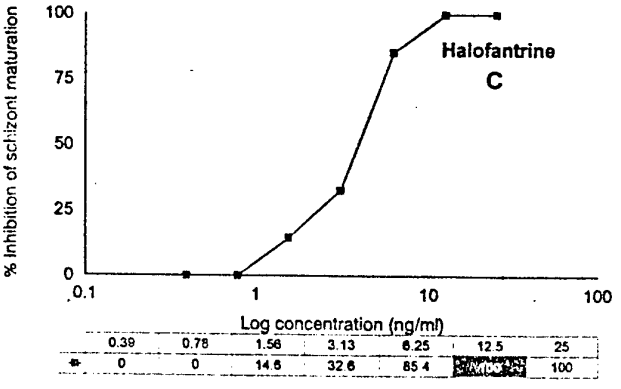
Fig. 12B: Effect of initial parasitaemia on MIC of chloroquine against P.knowlesi in vitro.











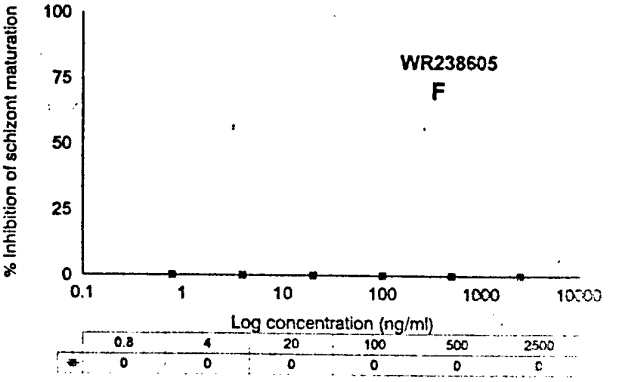
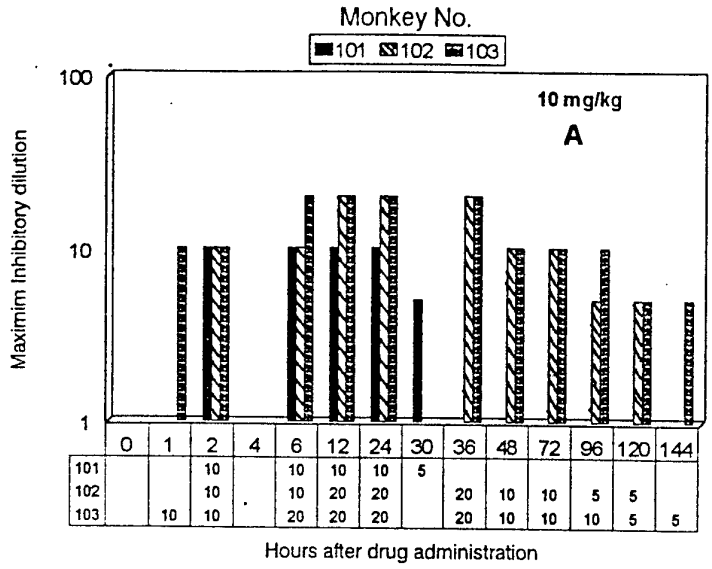
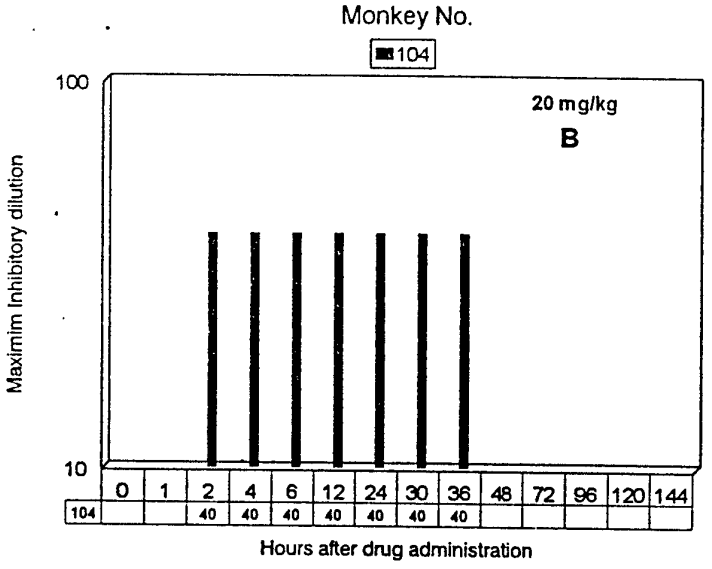


Fig. 13 *Drug sensitivity assay of antimalarials against P. knowlesi*





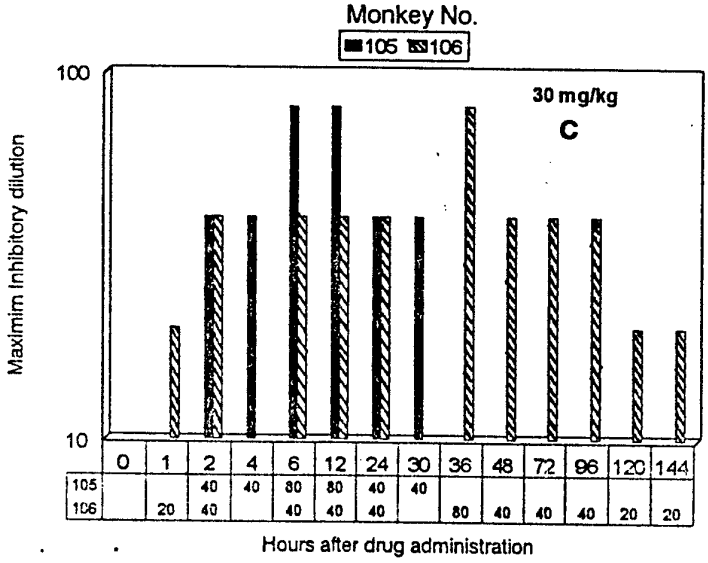


Fig.14 : Bioassay of Maximum Inhibitory Dilution of sequential serum samples from Chloroquine treated rhesus monkeys against *P. knowlesi*

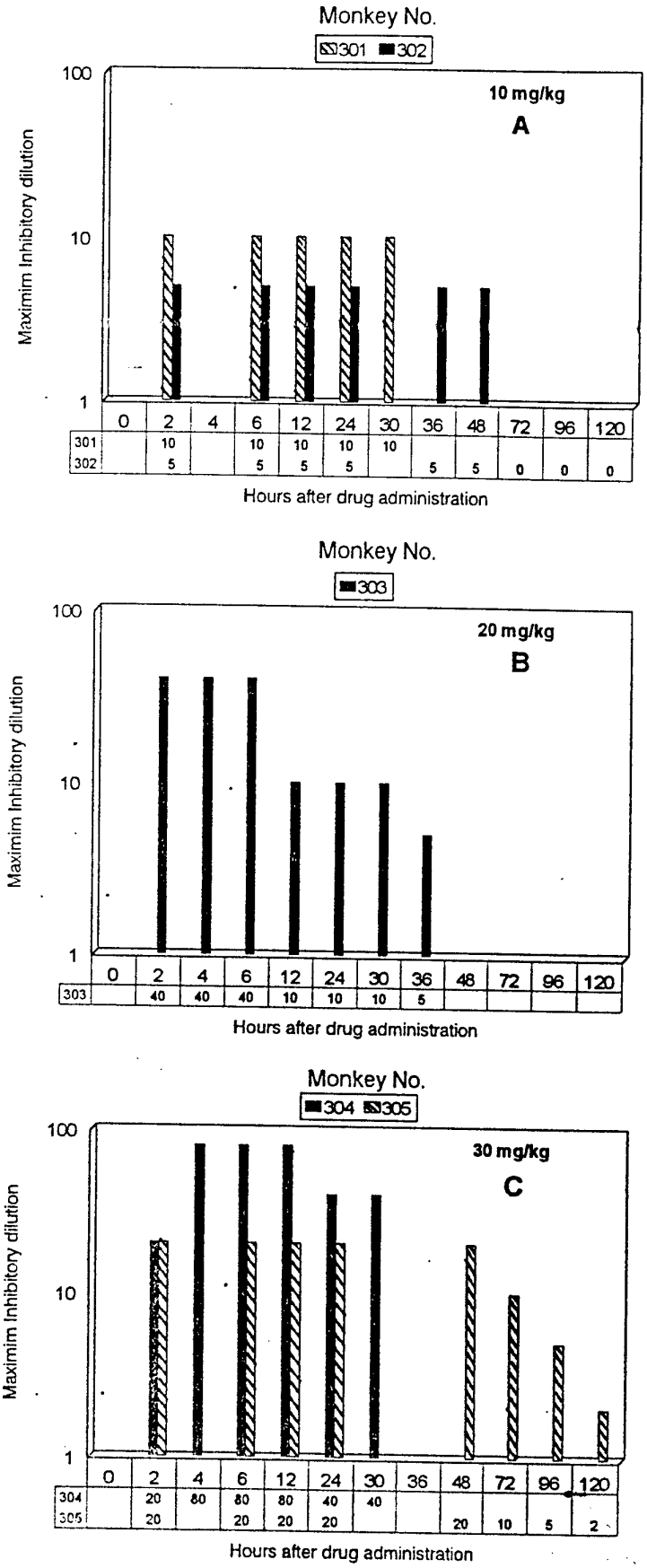
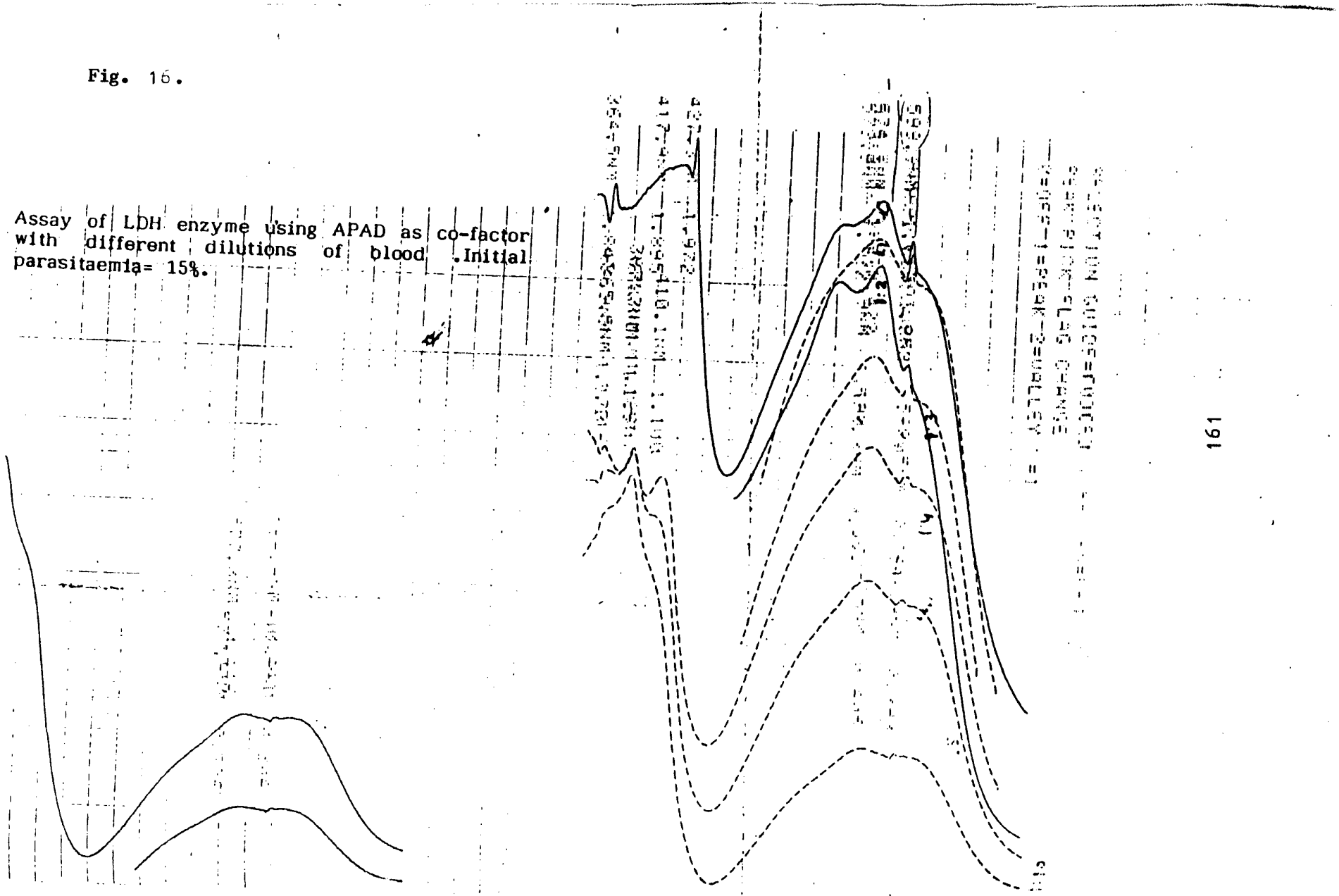


Fig. 15 : Bioassay of Maximum Inhibitory Dilution of sequential serum samples from Halofantrine treated rhesus monkeys against *P. knowlesi*

Fig. 16.

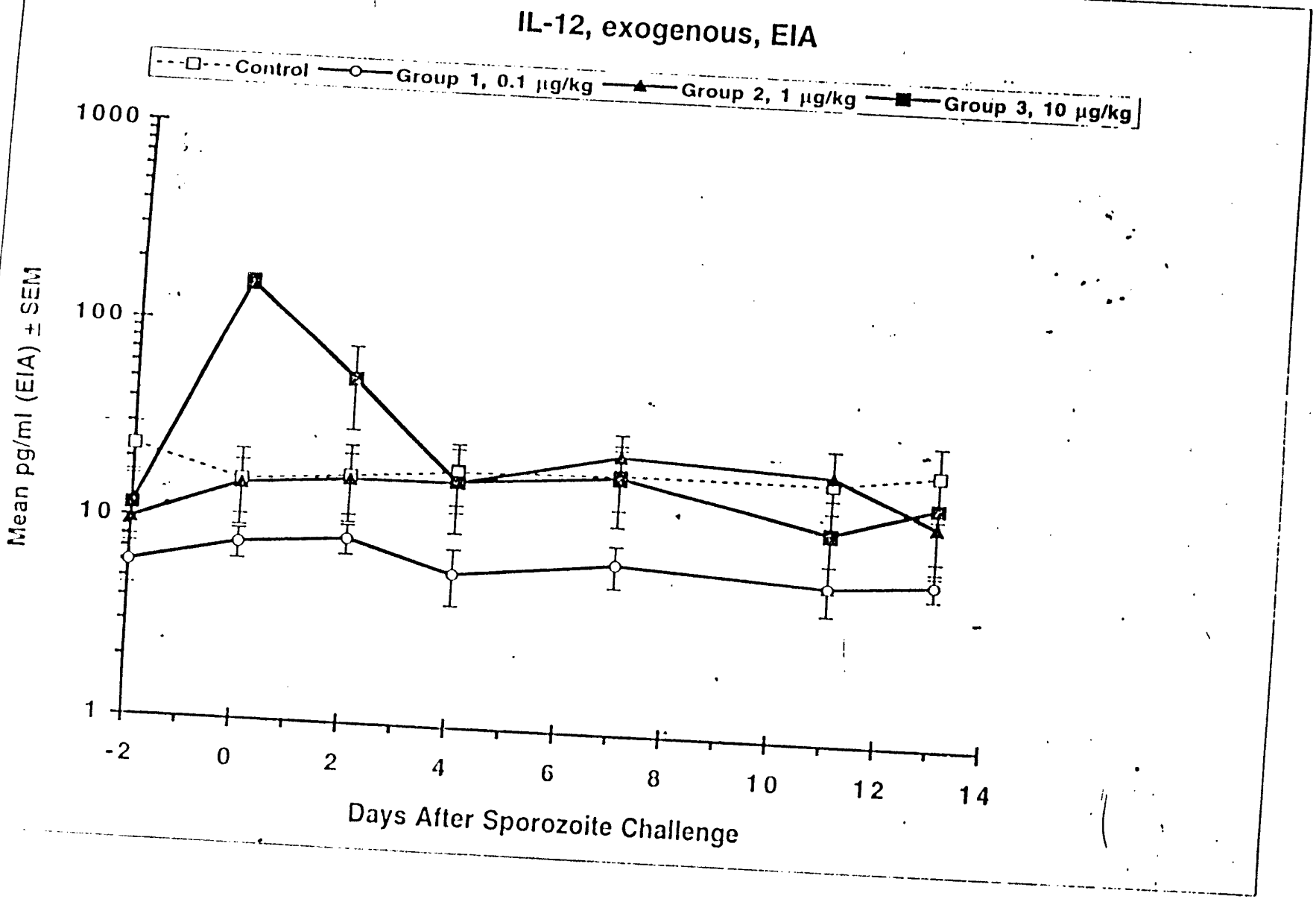
Assay of LDH enzyme using APAD as co-factor with different dilutions of blood .Initial parasitaemia= 15%.

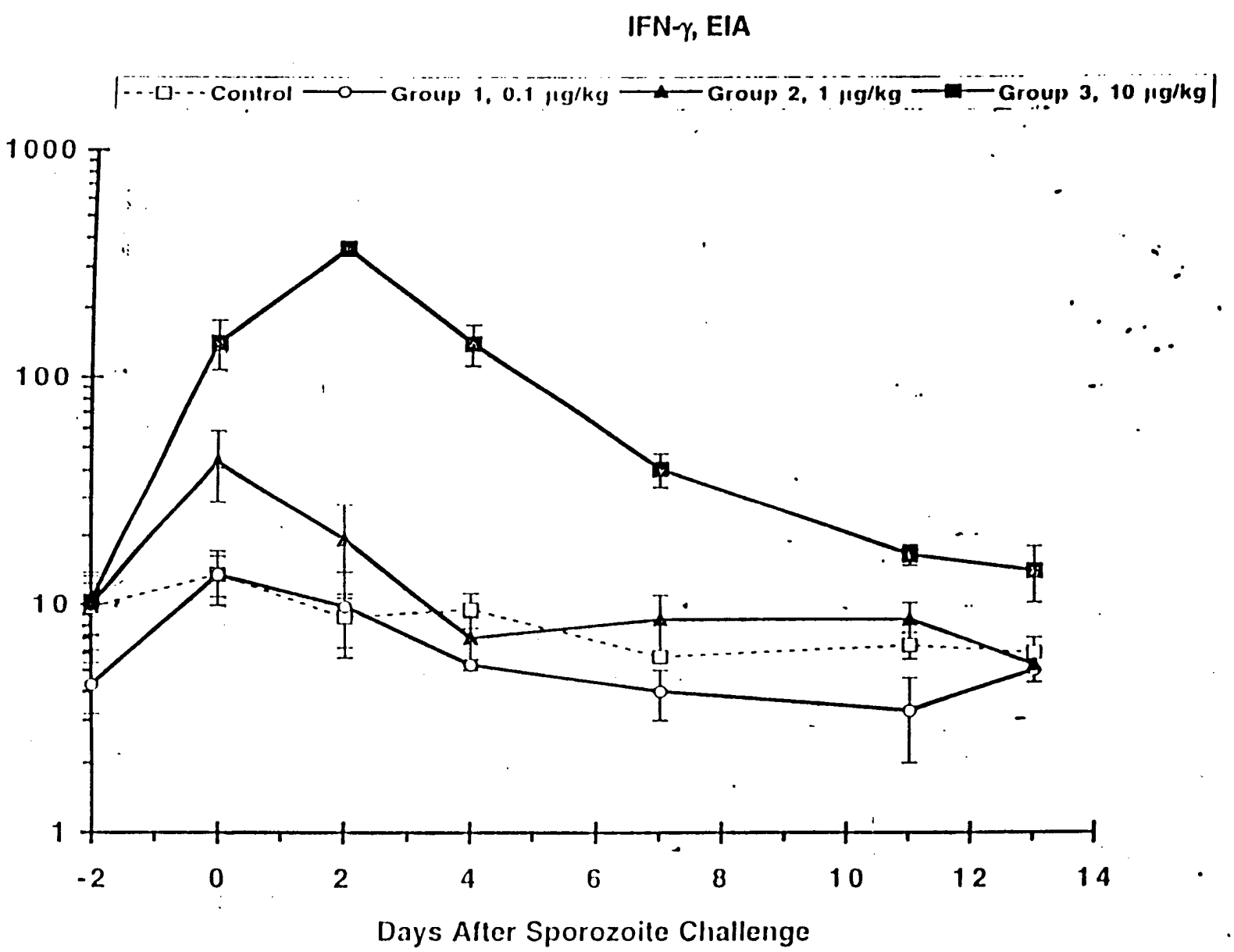


Normal mouse blood

P.yoelii infected blood.

Fig. 17


IL-12, exogenous, EIA
Control
Group 1, 0.1 µg/kg
Group 2, 1 µg/kg
Group 3, 10 µg/kg
Mean pg/ml (EIA) ± SEM
1000
100
10
1
-2
0
2
4
6
8
10
12
14
Days After Sporozoite Challenge


IFN-γ, EIA
Control
Group 1, 0.1 µg/kg
Group 2, 1 µg/kg
Group 3, 10 µg/kg
1000
100
10
1
-2
0
2
4
6
8
10
12
14
Days After Sporozoite Challenge